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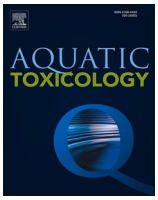

The toxicity and toxicokinetics of imidacloprid and a bioactive metabolite to two aquatic arthropod species

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ABSTRACT

Previous studies have explored effects of imidacloprid and its metabolites on terrestrial species, such as bees, and indicated the importance of some active metabolites. However, the biotransformation of IMI and the toxicity of its metabolites to aquatic arthropods are largely unknown, especially the mechanisms driving species sensitivity differences and time-cumulative toxicity effects. To assess the potential effects of the metabolization of IMI and the toxicokinetics and toxicity of the metabolite(s) on aquatic arthropods, we first studied the acute toxicity of IMI and relevant metabolites to the mayfly species *Cloen dipterum* (sensitive to IMI) and the amphipod species *Gammarus pulex* (less sensitive to IMI). Secondly, toxicokinetic experiments were conducted using both the parent compound and imidacloprid-olefin (IMI-ole), a metabolite assessed as toxic in the acute tests and defined as bioactive. Of the four tested metabolites, only IMI-ole was readily biotransformed from the parent IMI and showed similar toxicity to *C. dipterum* as IMI. However, *C. dipterum* was hardly able to eliminate IMI-ole from its body. For *G. pulex*, IMI-ole was also the only detected metabolite causing toxicity, but the biotransformation of IMI to IMI-ole was slower and lower in *G. pulex* compared to *C. dipterum,* and *G. pulex* eliminated IMI-ole quicker than *C. dipterum*. Our results on internal kinetics of IMI and IMI-ole, and on biotransformation of IMI indicated that the metabolite IMI-ole was toxic and was rather persistent inside the body tissue of both invertebrate species, especially for *C. dipterum*. In conclusion, as IMI and IMI-ole have similar toxicity and IMI was replaced rapidly by IMI-ole which in turn was poorly eliminated by *C. dipterum*, the overall toxicity is a function of dose and time. As a result, no long-term threshold of effects of IMI may exist for *C. dipterum* as the poor elimination results in an ongoing increase of toxicity over time for mayflies as also found experimentally in previous published papers.

1. Introduction

Neonicotinoids (NNIs) are a class of neuro-active insecticides acting in a similar manner as nicotine (Hladik et al., 2018), comprising different families i.e. the nitroguanidine insecticides (imidacloprid) and the cyanoguanidine insecticides (thiacloprid). They are used worldwide and spark increasing environmental concerns due to their potential risks to terrestrial and aquatic invertebrates (Hladik et al., 2018; Morrissey et al., 2015). Imidacloprid (IMI) is the most used NNI, and the toxicity of IMI has been extensively studied for terrestrial insects, like bees (Suchail. et al., 2001; Zaworra et al., 2019). As IMI gets into freshwater ecosystems readily and is relatively persistent, there are some aspects regarding IMI that need to be studied in order to fully understand its long-term toxicity to aquatic invertebrates (Morrissey et al., 2015; Starner and Goh, 2012; Vijver and van den Brink, 2014). The first one is the species sensitivity differences. For example, mayflies are amongst the most sensitive species to IMI with 96h-EC_{50} and 28d-EC_{50} values being 18 and 122 times lower than those of *G. pulex*, which is a macroinvertebrate with an average acute sensitivity to IMI (Ashauer et al., 2011b; Roessink et al., 2013). The second one is the time-dependant increase of the effects of IMI for sensitive species, such as bees (Rondeau et al., 2014) and mayflies (Van den Brink et al., 2016) after a long term exposure. For example, the toxicity of IMI to *C. dipterum* increased from a 4d-EC_{50} value of 1.02 μg/L to a 28d-EC50 of 0.126 μg/L, while for the less sensitive *G. pulex* this shifted from a 4d-EC_{50} value of 18.3 μg/L to a 28d-EC_{50} of 15.4 μg/L (Roessink et al., 2013)

Studies have revealed that traits, such as organism size, respiration mode and lipid content (Rubach et al., 2010), as well as the formation of

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metabolites can be possible explanations for species specific toxicity (Fu et al., 2018; Kretschmann et al., 2011; McCarty et al., 2011). That metabolites of IMI, such as 5-hydroxyimidacloprid and olefin IMI, result in delayed and time-cumulative toxicity of imidacloprid has been propounded in some terrestrial studies (Suchail. et al., 2001; Tomalski et al., 2010). Furthermore, some studies have demonstrated that some metabolites of IMI display similar or even higher neurotoxicity than the parent compound in locust (Parkinson and Gray, 2019) and bees (Nauen et al., 2001), but these studies exclusively focused on terrestrial species. In line with the previous recommendation (Starner and Goh, 2012) of monitoring IMI metabolites, like imidacloprid-olefin (IMI-ole) and hydro imidacloprid in surface waters, a recent study detected IMI and its metabolites in source and tap water (Wan et al., 2020). Besides metabolites being present in the surface waters, organisms also can take IMI up and biotransform it, as demonstrated in several species like rock oysters (Ewere et al., 2019), lizards (Wang. et al., 2019) and bees (Suchail et al., 2004a, 2004b; Suchail. et al., 2001). In spite of the fact that metabolic pathways may be species-specific, one of the common pathways is that IMI is transformed to 5-OH-IMI, to IMI-ole and to 6-CNA (Fusetto et al., 2017; Li et al., 2019; Nishiwaki et al., 2004). To understand the effect pathway of IMI and its metabolites in aquatic invertebrates, we chose IMI and its metabolites imidacloprid-olefin, imidacloprid-urea, 6-chloronicotinic acid, and 5-hydroxy-imidacloprid in our study based on their commercial availability their potential toxicity (Fusetto et al., 2017; Li et al., 2019; Ma et al., 2014; Nishiwaki et al., 2004; Suchail et al., 2004a; Tomalski et al., 2010) and environmental occurrence (Benton et al., 2015; Wan et al., 2020).

To increase the understanding of the mechanism of toxicity and its increase with time, a toxicokinetic-toxicodynamic (TKTD) model, e.g. belonging to the General Unified Threshold model for Survival (GUTS) can be used (Ashauer et al., 2010). The TK part of the model describes the fate of a toxicant from the surrounding environment to the internal body and may consist of uptake, absorption, excretion, and biotransformation (Kretschmann et al., 2011), depending on the complexity of the model used. The TD part of the GUTS framework usually models survival, but, immobility without recovery can also be modelled by the GUTS model (Ashauer et al., 2011a; Jager et al., 2011). Immobility is a better endpoint for IMI since neonicotinoids are neurotoxic and expected to cause effects on behaviour (Morrissey et al., 2015). Currently, some studies are available which include bioaccumulation and metabolisation of IMI in bees (Nauen et al., 2001; Suchail et al., 2004a; Zaworra et al., 2019), plants (Mach et al., 2018), and lizards (Wang et al., 2018), while one study with internal concentration measurements is available for aquatic organisms (Ashauer et al., 2010). In that study, no metabolization of the parent compound was observed in *G. pulex*. However, *G. pulex* may not be the most representative aquatic organism for IMI, as it is relatively insensitive compared to aquatic insect species (Roessink et al., 2013; Van den Brink et al., 2016).

To understand the mechanism of IMI toxicity to aquatic organisms and the roles of its metabolites, the current study was designed to address (1) whether the metabolites are similarly toxic as IMI to sensitive and less-sensitive species, using *C. dipterum* and *G. pulex* as representatives; (2) whether toxicokinetics (TK) of IMI and its toxic metabolites, can explain the difference in toxicity of IMI to two different species as well as the observed increase of toxicity with time. To address this, two types of experiments were performed. First, the toxicity of IMI and different metabolites was assessed by an acute standard toxicity experiment. Secondly, kinetic and bioconcentration experiments were conducted and a one compartment TK model and biotransformation TK model approaches were used to address the necessity of including biotransformation in the assessment of the species specific overall risks of IMI. Besides, a full GUTS model was used to predict the long term effects of IMI and IMI-ole to both species.

2. Materials and methods

2.1. Chemicals and test organisms

Imidacloprid (IMI; CAS: 138261–41–3) and its commercial available metabolites imidacloprid-olefin (IMI-ole; CAS: 115086–54–9), 5-hydroxy-imidacloprid (5-OH-IMI; CAS: 155802–61–2), imidacloprid-urea (IMI-urea; CAS: 120868-66-8) and 6-chloronicotinic acid (6-CNA; CAS: 5326–23–8) were used in our experiments (Table S1). Imidacloprid-d4 (IMI-d4; CAS: 1015855–75–0) was used as an internal standard during the analytic measurements of any organism samples.

The stock solutions of IMI (200 μg/mL) and IMI-d4 (200 μg/mL) were dissolved into 2% acetone (v: v) to ensure that the compounds were fully dissolved. The volume percentage of acetone in the experimental jars was less than 0.01% (v: v). IMI-ole, IMI-urea, 5-OH-IMI and 6-CNA were dissolved into MiliQ water.

Two species, the ephemeropteran *Cloeon dipterum* and the amphipod *Gammarus pulex*, were used in the experiments. *C. dipterum* was collected from an uncontaminated test system at the outdoor research site 'De Sinderhoeve' located in Renkum, the Netherlands (www.sinderhoeve.org). *G. pulex* was collected from an uncontaminated location, the Heelsumse Beek (a brook with the coordinates 51.973400, 5.748697). After collection, the organisms were kept in aerated tanks in the laboratory for at least 3 days to acclimate them to laboratory conditions. During the acclimation period, organisms were fed ad libitum with biofilm, organic matter and periphytic algae for *C. dipterum* and leached *Populus* leaves for *G. pulex* (Roessink et al., 2013). All jars containing the test organisms were placed in a water bath maintained at 18 ± 1 °C with a light regime of 12:12 h light: dark. The light in our water bath did not contain ultraviolet light in order to prevent the photodegradation of IMI and its metabolites which was confirmed by the analytical measurement with LC/MS-MS. The groundwater obtained from the Sinderhoeve experimental station and also the freshwater from the organisms collection locations have been evaluated by LC/MS-MS to confirm the lack of presence of all the tested analytes.

In addition to the number of organisms required for the toxicity experiments, approximately 50 extra organisms were collected at the beginning of each experiment to characterize the test population by measuring the length and lipid content of the individuals using methods described by Rubach and co-workers (Rubach et al., 2010).

2.2. Toxicity experiments

The acute toxicity of each compound (IMI, IMI-ole, IMI-urea, 6-CNA and 5-OH-IMI) was assessed by a 4-day standard acute toxicity test to assess, the EC_{50} and LC_{50} for *C. dipterum* and *G. pulex* (Roessink et al., 2013). In detail, separate tests were performed on each test compound and species combination. Per replicate system, 12 mayflies were placed in 1 L groundwater obtained from the Sinderhoeve experimental station. The volume was dosed with its respective compound to reach final exposure concentrations of 0, 1, 10, 30, 100 or 300 μg/L. The tests were performed between December 2018 and February 2019, using a winter generation of *C. dipterum*. For the tests with *G. pulex*, a replicate test system consisted of 10 individuals in 1 L groundwater, after which, the volume was dosed to reach concentrations of 0, 10, 30, 100, 300 or 1000 μg/L. These tests were performed in July 2019, using a summer generation of *G. pulex*. Experiments were performed with three replicates per treatment level, while five replicates were used for control and the solvent control. The test systems were not aerated during the experiments to minimize evaporation of chemicals, and the dissolved oxygen content was acceptable with a value of higher than 7 mg/L at the end of experiment (*SI.xlsx*). In the experiments with *G. pulex,* a piece of stainless steel mesh was added to provide shelter. Organisms were checked every day and the effect status (dead, immobile or mobile) of each individual organism was assessed according to Roessink et al. (2013). Dead and immobilised organisms were both considered as

immobile. Dead organisms and moults were removed daily. 1 mL of water was taken daily to verify the exposure concentration dynamics of the spiked chemical and to measure the concentration of the tested analytes (IMI, 5-OH-IMI, IMI-urea, IMI-ole, 6-CNA) using LC-MS/MS (see Section 2.5). All jars were placed randomly in a water bath, temperature and light conditions were the same as the acclimation period. When control immobility exceeded 10%, the results of the whole experiment were considered to be indicative only (Roessink et al., 2013). Dissolved oxygen, pH, electrical conductivity, and temperature were measured at the start and end of the test in the control group and the highest treatment only, and the results are provided in the support information (*SI.xlsx*).

2.3. Toxicokinetic experiments

In the toxicokinetic experiments, *C. dipterum* and *G. pulex* were both exposed to IMI and IMI-ole at a concentration lower than one-tenth of the 48h-EC_{50} for immobilisation, as determined in the toxicity experiments, to ensure that no effects of the chemicals would occur on tested organisms. Exposure lasted for 2 days, after which organisms were transferred to new groundwater for a 3 days depuration period. In detail, for the tests with *C. dipterum*, 18 individuals were put in 1 L groundwater and exposed to 6.4 μg/L IMI or 5.4 μg/L IMI-ole. For the tests with *G. pulex*, 10 individuals were put in 1 L groundwater containing 12.5 μg/L IMI or 7.9 μg/L IMI-ole. After 2 days of uptake, the alive organisms were rinsed for 30 s using clean MillQ water, and transferred to 1 L clean groundwater to start the 3 days elimination phase. At around 0, 4, 9, 20, 25, 32, 48, 53, 74, 94, 104 and 120 h, 1 mL water was collected from the jars and analysed by LC-MS/MS (for detailed timepoints, see the raw data in *SI.xlsx*). For the TK experiments with IMI and *C. dipterum* and *G. pulex* and IMI-ole and *C. dipterum*, three replicates were sampled at each timepoint while five replicates were used for the experiments with *G. pulex* and IMI-ole. Negative controls were added with five replicates containing organisms but no chemical or solvent, five replicates contained organisms and solvent but no chemical and three replicates contained the chemical but not the organisms. From the sampled jars all alive organisms were collected, washed by MillQ water for 30 s, and stored at −20 °C for further chemical analysis. The concentration of IMI and IMI-ole, and the possible other analytes were measured in both the organisms and the water during the uptake and elimination phase. All remaining organisms were checked for status every day and the dead organisms and moults were removed daily.

2.4. Bioconcentration of IMI and the generation of its metabolites

To explore the contribution of the passive absorption of IMI to the body surface of the dead organisms, organisms collected in September 2019 were immediately frozen after collection (−20 °C, for 24 h). After this, the organisms were thawed and added to groundwater containing 24 or 120 μg/L IMI in case of *C. dipterum* and to 240 or 1200 μg/L in case of *G. pulex*. The treatments and solvent control had three replicates. After 24 h, all the organisms were taken out and rinsed using clean MillQ water. The internal concentration through passive absorption will be compared to the internal concentrations measured in organisms which died on day 1 at the same concentration of exposure (see below).

In order to facilitate the detection of metabolites and also compare the bioconcentration ratio of IMI amongst different status (dead, immobile, mobile) of organisms after 4 days, we exposed the two species to a higher exposure concentration than in the TK experiment. These two higher concentrations intended to cause 50% and 100% immobilization and 20% and 50% death based on previous acute toxicity results. The identification of organisms status was the same as in the acute toxicity experiments. After 4 days of exposure, the internal concentration of IMI and its metabolites was measured. In detail, 12 individuals of *C. dipterum* and 10 individuals of *G. pulex* collected in September 2019 were exposed in 1 L groundwater to 24 or 120 μg/L IMI in case of *C. dipterum* and to 240 or 1200 μg/L in case of *G. pulex*. Each treatment had six replicates while the control and solvent control contained five replicates. Dead organisms were removed every day and were stored in the freezer (−20 °C) for further analysis. After 4 days of exposure, the organisms were taken out from the jars and washed by clean water for 30 s. In order to evaluate to what extent the passive physical absorption can account for the estimated bioaccumulation, the internal concentration of the organisms which died during the first day of experiment will be compared with the passive absorption of the corpse organisms which were dead before exposure. For the other organisms, they were classified as dead, immobile or mobile and analysed for their internal concentration of IMI and the potential metabolites. When on a certain sampling date, only a few organisms died in one replicate, the dead organisms of different replicates within a treatment level were merged together to achieve enough material for the analysis of the internal concentration. Hence, the replication of the internal concentration results varied from 2 to 6 (for detailed information of replication, see the raw data in *SI.xlsx)*. The results were used to calculate the bioconcentration ratio (BCR) of IMI and the generation ratio of potential metabolites. The analytical verification of the concentrations can be found in Section 2.5.

2.5. Chemical analysis

In the toxicokinetic experiment with IMI and IMI-ole (Section 2.3) and the bioconcentration of IMI and the generation of its metabolites (Section 2.4), the internal concentration of parent compounds and the potential metabolites (IMI-ole, 5-OH-IMI, IMI-urea, 6-CNA) were measured at each timepoint in each test. For the analytical quantification of the concentrations, all samples were taken out of the freezer and the organisms were lyophilised for 1 day and weighted to get the dry weight of animals. 1 mL 1% acetic acid MeOH: Water (v: v = 5: 1) extraction solution and 25 μL internal standard (imidacloprid-d4, 200 μg/L) were added. Then the samples were homogenized with a Minilys personal homogeniser (Bertin Instruments, France) using a Precellys ceramic lysing kit (1.4/2.8 mm; Bertin Instruments, France) for 3 times 60 s at 3000 rpm using a 30 s interval in between. After this, the sample was centrifugated at 10,000 rpm at 10 min, and the supernatant was filtered over a PTFE syringe filter (pore size 0.45 μm), into a 2 mL injection vial. Filters were injected with 200 μL extraction solution again to regain the chemical may remain on the filter, this filtrate in turn, was centrifugated and filtered over a syringe filter (0.45 μm) as well. Afterwards, the two filtrates were combined and a final volume of 1.2 mL was collected, after which the sample was ready for analysis by LC-MS/MS. The water samples were analysed directly, without an extraction step.

All samples were analysed by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kamel, 2010; Roessink et al., 2013). The analyses were performed on an Agilent 1260 Infinity liquid chromatography coupled with a 6460 Triple quad mass spectrometer (Agilent Technologies, USA). Separations were carried out on an Agilent Eclipse Plus C18 column (4.6 × 150 mm, 5 μm) at 40 °C. The injection volume of the samples was set at 30 μL. The mobile phase used was MeOH + 0.1% Formic acid (C) and Milli-Q water+ 0.1% Formic acid (D) with the following multistep gradient: 0–1.5 min: 90/10 (C/D, v:v); 1.5–2.5 min: 90/10 (C/D, v:v) to 50/50 (C/D, v:v); 8 min: 50/50 (C/D, v:v); 8–8.1 min: 50/50 (C/D, v:v) to 0/100 (C/D, v:v); 9 min: 0/100 (C/D, v:v); 9–9.1 min: 0/100 (C/D, v:v) to 90/10 (C/D, v:v); 9.1–12 min: 90/10 (C/D, v:v) at a flow rate of 0.7 mL/min. The mass spectrometer was operated using Agilent jet stream electrospray ionization source (AJS-ESI) in positive mode. Nitrogen was used both as nebulizer and collision gas, the capillary voltage was 5000 V and the temperature of the ion source (TEM) was set at 300 °C. The compounds were detected in multiple reaction monitoring

(MRM) using two transitions per compound. The MS/MS transitions of all compounds are provided in Table S2.

Injected samples were quantified by peak area using the calibration curve constructed from calibration standards included in the same sample sequence. Agilent Masshunter software (version 7.0) was used for instrument control and data acquisition. The extraction recovery of each tested analytes (IMI, IMI-ole, 5-OH-IMI, IMI-urea, 6-CNA) in the organisms, evaluated at two concentrations by spiking them into the clean organisms, were acceptable for both species based on recovery and repeatability (for further information of analysis methods and recovery results, see Text S2 and Table S3, S4, S5 in Support information).

2.6. TK and GUTS modelling

2.6.1. First-order one compartment kinetic model

To determine the toxicokinetic rate constants of uptake and elimination for IMI and its metabolite IMI-ole, a first-order compartment kinetic model programmed in Matlab R2018b (http://www.debtox.info/byom.html) was used. The script was based on the method of byom_calanus_2016_onecomp in Acute Calanus package (*BYOM, version 1.1*), with a small modification that the lower limit of elimination rate was 0 instead of 0.1 in the original script.

The uptake and elimination rate of IMI and IMI-ole were estimated using a one-compartment, first-order kinetic model (Jager et al., 2011):

$$\frac{dC_{int}(t)}{dt} = k_{up}\cdot C_w(t) - k_{ep}\cdot C_{int}(t) \quad \text{(Eq. 1)}$$

with t = time; C_{int} =internal concentration of exposed compound; C_w = concentration of exposed compound in water, k_{up} = the uptake rate constant of parent (exposed) compound and k_{ep} = the elimination rate constant of parent (exposed) compound.

The Bioconcentration factor (BCF) ($L\bullet kg^{-1}$) was calculated by a kinetic method (BCF_k):

$$BCF_k = \frac{k_{up}}{k_{ep}} \quad \text{(Eq. 2)}$$

The elimination half-life ($t_{½}$) (day) was calculated from the elimination rate of IMI or IMI-ole:

$$t_{1/2} = \frac{ln2}{k_{ep}} \quad \text{(Eq. 3)}$$

2.6.2. Biotransformation TK model (Bio-TK)

For a better understanding of the biotransformation of IMI in the two species, the biotransformation model as described by Kretschmann et al. (2011) was used, with one minor modification. The modification was that in our model we considered IMI-ole as the only metabolite of IMI, the remaining potential unknown metabolites were all included in $b\_k_{e\_parent}$ (elimination rate) of IMI. The model script was based on the method of byom_bioconc_start (BYOM, version 5.2) with the modifications that we introduced the biotransformation parameter, $b\_k_{bio\_parent}$ (Eq. 4) and the elimination rate of metabolite $k_{e\_metabolite}$ (Eq. 5). To better distinguish the two model approaches, parameters in this biotransformation model have a prefix of b.

The uptake and elimination rate of IMI, as well as its biotransformation rate, was estimated using a one-compartment, first-order kinetic model:

$$\frac{dC_{int_{parent}}(t)}{dt} = b\_k_{u\_total}\cdot C_w(t) - b\_k_{e\_parent}\cdot C_{int_{parent}}(t) - b\_k_{bio\_parent}\cdot C_{int_{parent}}(t) \quad \text{(Eq. 4)}$$

with t = time; $C_{int_{parent}}$ = internal concentration of IMI, C_w= water concentration of IMI, $b\_k_{u\_total}$= the uptake rate constant for IMI, $b\_k_{e\_parent}$= the elimination rate constant of IMI and $b\_k_{bio\_parent}$ = the biotransformation rate constant of IMI to IMI-ole.

For the metabolite (IMI-ole) the TK model used was:

$$\frac{dC_{int_{metabolite}}(t)}{dt} = b\_k_{bio\_parent}\cdot C_{int_{parent}}(t) - b\_k_{e\_metabolite}\cdot C_{int_{metabolite}} \quad \text{(Eq. 5)}$$

with t = time; $C_{int\_metabolite}$ = internal concentration of IMI-ole, $C_{int_{parent}}$=internal concentration of IMI, $b\_k_{bio\_parent}$ = the biotransformation rate constant of IMI to IMI-ole and $b\_k_{e\_metabolite}$ = the elimination rate constant of IMI-ole.

The total elimination rate ($b\_k_{e_{total}}$) of IMI was:

$$b\_k_{e_{total}} = b\_k_{bio\_parent} + b\_k_{e\_parent} \quad \text{(Eq. 6)}$$

The BCF kinetic of IMI (BCF_k) was calculated as:

$$b\_BCF_k = \frac{b\_k_{u\_total}}{b\_k_{e_{total}}} \quad \text{(Eq. 7)}$$

Elimination half-life ($t_{½IMI}$) (day) was calculated from the elimination rate for IMI:

$$b\_t_{1/2IMI} = \frac{ln2}{b\_k_{e_{total}}} \quad \text{(Eq. 8)}$$

2.6.3. Prediction of long term effects of each compound by the GUTS model

The GUTS model approach includes a reduced model and a full model depending on whether the measured internal concentration is included (Jager et al., 2011). Besides, GUTS uses individual tolerance (IT) and stochastic death (SD) concepts to describe the effect mechanism on the endpoint (Jager et al., 2011). IT assumes that organism die (or is immobilized) immediately on reaching a critical internal concentration, which is unique for each individual. SD assumes that all individuals are identical but mortality is a probabilistic process, which increases with increasing internal concentration of the substance (Van den Brink et al., 2013). Both are rational in explaining the effect mechanism, and this also illustrates the mechanism diversity of organisms exposed to different substances (Ashauer et al., 2016).

Both the reduced and the full GUTS models were used to describe the TKTD of IMI and IMI-ole in the two species. The models used were similar to the ones used by Jager and co-authors (Jager et al., 2017), with two minor modifications. First, we used the mobility fraction instead of the survival fraction, second, we set the lipid content percentage to zero, as none of the analytes in our tests are hydrophobic. For specific model scripts and optimization used, see the Acute Calanus package (*BYOM, version 1.1*)(Jager et al., 2017). We calibrated both the reduced and full GUTS models including both the IT and SD mechanisms to our data sets, so 4 models in total were parameterised. amongst the models for each data set, the Akaike information criterion (AIC) and goodness-of-fit measures were used to select the best-fitting model. This is not a formal significance test but has been broadly used to select alternative models (Anderson., 2002). A difference of AIC of more than 6 can be interpreted as that the poorest model is 0.05 times as probable as the best model to minimise information loss (Jager et al., 2017).

2.7. Statistics

Dose-response relationships were fitted using the nominal exposure concentration at the endpoints (mortality and immobilisation) with acute toxicity data using the following log-logistic equation, calculated using Genstat 19th edition (VSN International Ltd).

$$y(conc) = \frac{1-c}{1+e^{-b\times(\ln conc - a)}} \quad \text{(Eq. 9)}$$

Where y is the fraction of dead or immobile test organisms, conc is the concentration (μg/L), a is ln(median effect concentration [EC_{50}]) or ln(median lethal concentration [LC_{50}]) (μg/L), b is the slope (L/μg), and c is the fraction of control mortality or immobilisation(-) (Roessink et al., 2013).

As the steady state may not be reached within 4 days, especially in the higher exposure concentrations, the bioconcentration ratio (BCR) was used to describe the internal concentration of IMI (Eq. 10), and the generation ratio was used to describe the metabolite, IMI-ole (Eq. 11).

$$\text{Bioconcentration ratio of IMI} = \frac{\text{internal concentration of IMI}}{\text{exposure water concentration}} \quad \text{(Eq. 10)}$$

$$\text{IMI} - \text{ole generation ratio} = \frac{\text{internal concentration of IMI} - \text{ole}}{\text{internal concentration of IMI} - \text{ole} + \text{internal concentration of IMI}} \quad \text{(Eq. 11)}$$

Significant differences between treatments were assessed using R (version 3.5.1). The assumptions of normality were assessed with a Shapiro-Wilk test, and the assumption of equal variance was assessed using a Spearman rank correlation between the residuals and dependant variable. If assumptions of normality and equal variance were passed, a one-way analysis of variance (ANOVA) with α=0.05 and a post-hoc Tukey's test was conducted. If assumptions failed, a Kruskal-Wallis test, with α=0.05, and a post-hoc Dunn's test was used.

3. Results and discussion

3.1. Sensitivity of IMI and its four metabolites to C. dipterum and G. pulex

All measured exposure concentrations at the start and the end of the test were within 10% variation (+,-) from nominal concentrations, thus the nominal concentrations of each analyte (IMI, IMI-ole, IMI-urea, 5-OH-IMI and 6-CNA) were used for all calculations. The control immobility of all tests was within 10%, therefore the results were considered reliable. Amongst the five analytes, only IMI and IMI-ole showed effects to the tested species within the test concentration range, therefore we defined that IMI-ole is the bioactive metabolite. Therefore, only the results of IMI and IMI-ole will be presented and discussed. All other analytes showed no toxicity at concentration levels $\leq$ 300 µg/L to *C. dipterum* and $\leq$ 1000 µg/L to *G. pulex*.

Based on the 96h-EC_{50} values, IMI was a factor of 2.3 more toxic to *C. dipterum* than IMI-ole, while their 96h-LC_{50} were close (Table 1). The 96h-EC_{50} of IMI in our study was comparable to the 96h-EC_{50} value of 18 µg/L, reported by Van den Brink and co-workers (Van den Brink et al., 2016) for a winter generation of *C. dipterum*. Although the toxicity of IMI-ole to *C. dipterum* was somewhat delayed at the start (day 1) compared to IMI it reached similar toxicity in terms of lethality compared to IMI on day 4 (96h-LC_{50} for IMI was 90.6 µg/L and for IMI-ole 104 µg/L, Table 1). Our results agree with a study performed by Suchail et al. (2001), where an oral application IMI-ole exerted similar acute toxicity to honeybees as IMI, while two other imidacloprid metabolites (IMI-urea, 6-CNA) showed no or much lower toxicity

Similar to *C. dipterum*, only IMI and IMI-ole showed a 96h-EC_{50} < 1000 µg/L for *G. pulex* (Table 1). IMI-ole was 4.2 times less toxic than IMI in terms of 96h-EC_{50} (Table 1). More specifically, the 96h-EC_{50} of IMI and IMI-ole to *G. pulex* were 109 and 456 µg/L, respectively, while the 96h-LC_{50} were 731 and > 1000 µg/L, respectively (Table 1). Our results for IMI to *G. pulex* are in line with those of Ashauer et al. (2011b) and Van den Brink et al. (2016), who reported 96h-EC_{50} values of 132 µg/L and 49 µg/L, respectively.

In addition, we measured all analytes (IMI, IMI-ole, 5-OH-IMI, IMI-urea, 6-CNA) in all water samples of all treatments. We only found 6-CNA in the water samples of the highest IMI-ole treatment and the percentage of 6-CNA to IMI-ole was about 2% - 3% for both species (Figure S1). Although no other aquatic study is available, our finding is indirectly supported by studies that found 6-CNA under IMI exposure in bees (Suchail et al., 2004a) and in plants and soil (Li et al., 2019). Since 6-CNA is highly water soluble and quite stable in the environment (Zabar et al., 2011), depuration from aquatic organisms is likely to be quick. This could explain why we could not detect it in the tested organisms but only in water. As honeybees are not surrounded by water, 6-CNA may be less effectively excreted by this species, explaining why the metabolite was still detected in the bees (Suchail et al., 2004a).

To the best of our knowledge, our study is the first one showing toxic effects of the metabolites of IMI on aquatic invertebrates. Our results show a similar toxicity of IMI and IMI-ole to mayflies as also has been recorded for bees (Suchail. et al., 2001; Zaworra et al., 2019). This may imply that the some toxicokinetic and toxicodynamic processes underlying the toxicity of IMI and IMI-ole might be similar between aquatic and terrestrial insect species. It is probably a result from the fact that both the parent and the metabolite possess the nitroguanidine group, which binds tightly to the receptors in insects (Motohiro Tomizawa et al., 2000), IMI-ole showing a slightly greater affinity to nicotinic acetylcholine receptors (nAChRs) than IMI (Casida, 2011; Nauen et al., 2001), In contrast, the oral toxicity of 5-OH-IMI to honey bees is close to that of IMI (Nauen et al., 2001; Suchail. et al., 2001), while no effects of 5-OH-IMI could be detected in our study on either aquatic macroinvertebrate species. This absence of toxicity of 5-OH-IMI may firstly be explained by difference in exposure, as in our study we used waterborne exposure of 5-OH-IMI while oral feeding was used in the bee study. Secondly, although 5-OH-IMI also shares the same nitroguanidine group as IMI and IMI-ole, the receptor affinity of 5-OH-IMI is 8 times lower than IMI and 53 times lower than IMI-ole for bees (Nauen et al., 2001), and it showed a 13-fold lower binding affinity to whitefly nicotinic acetylcholine receptors (Rauch and Nauen, 2003).

Besides the receptor affinities, the difference in the toxicokinetics of imidacloprid and its metabolites in aquatic species could also explain the

Table 1.
The acute toxicity upon exposure of IMI and IMI-ole to C. dipterum and G. pulex provided in EC_{50} and LC_{50} values followed by the 95% confidence interval between brackets (an asterisk means that the CI could not be calculated) and the slope of the curve (parameter b) after the semicolon.

Tested Species	Exposure Compound	Day	1	2	3	4
C. dipterum	*IMI*	EC_{50}	109 (102 - 116); 16.0	86.2 (73.9 - 101); 11.8	40.9 (32.9 – 60.0); 4.06	24.4 (*); 11.1
		LC_{50}	311 (259 - 374); 4.01	110 (102- 117); 14.9	105 (99.4- 111); 15.6	90.6 (73.9–111); 4.06
	IMI-ole	EC_{50}	318 (252 - 401); 3.29	100 (*); 16.0	88.5 (*); 14.0	57.1 (43.1 – 75.6); 4.15
		LC_{50}	> 300	> 300	380 (149 – 971); 4.26	104 (98.6 - 110); 14.8
G. pulex	*IMI*	EC_{50}	170 (*); 2.49	145 (*); 2.82	165 (122 - 222); 2.30	109 (101 - 117); 14.0
		LC_{50}	> 1000	> 1000	> 1000	731 (290 - 1844); 0.587
	IMI-ole	EC_{50}	681 (533 - 870); 3.13	382 (270 - 542); 1.57	594 (449 - 784); 2.70	456 (350 - 595); 3.17
		LC_{50}	> 1000	> 1000	> 1000	> 1000

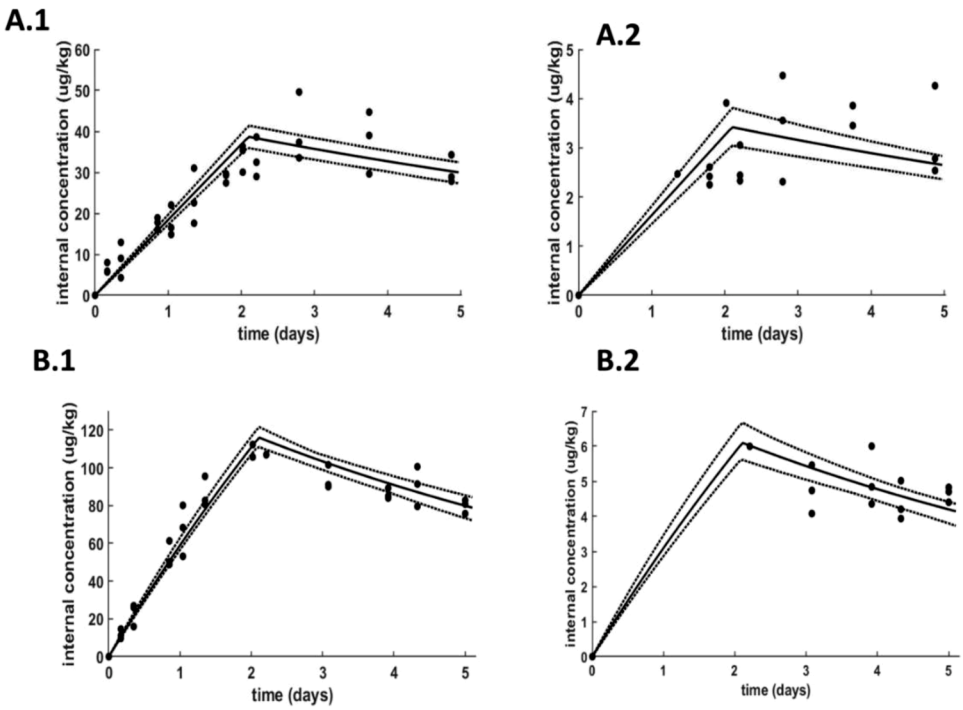

Fig. 1. The fit of the biotransformation TK model (bio-TK) for C. dipterum (A1: parent compound, IMI; A2: the metabolite, IMI-ole) and for G. pulex (B1: parent compound, IMI; B2: the metabolite, IMI-ole). The black lines represent the fit of the model generated by byom, the dotted line represented the confidence intervals of this fit while the dots represent the experimental results ($n = 3$ in A.1, A.2, B.1, B.2 with exception in Fig. A.2, at day 1.35, day 2.02, $n = 1$; in Figure A.2, at day 2.02, $n = 2$, day 2.21, $n = 1$).

differences in toxicity, which will be discussed below.

3.2. TK of IMI and IMI-ole in both C. dipterum and G. pulex

3.2.1. One compartment TK model approach of IMI and IMI-ole in both species

Both *C. dipterum* and *G. pulex* were exposed to IMI or IMI-ole for 2 days to study their uptake, and subsequently to clean water for 3 days to study their elimination (Fig. 1 and Fig. 2). In contrast to most studies that used a 1-day uptake period (Ashauer et al., 2010, 2012; Fu et al., 2018), we used a 2-days uptake period in our TK experiment. This is because the effects of IMI-ole in the acute toxicity study increased up to day 2 for both species (Table 1). The number of immobile organisms in all TK experiments was below 10%, confirming the initial assumption that the concentrations used were below acute toxicity levels. The water concentrations were stable during the uptake phase (*SI.xlsx*), and the average measured water concentration was used as the initial exposure concentration for each compound in each species. The change in fresh weight of the organisms overtime was not significant (Figure S2), showing that a five-day experiment without food did not result in weight loss, which was indicative of starvation.

We determined an uptake rate constant of IMI in *G. pulex* of 5.21 ($L \cdot kg_{ww}^{-1} \cdot d^{-1}$), which was about 2.5 times higher compared to the k_u of 1.96 $L \bullet kg_{ww}^{-1} \bullet d^{-1}$ determined by Ashauer et al. (2010) (Ashauer et al., 2010). They reported an IMI elimination rate constant of 0.27 (d^{-1}), while we calculated one of 0.12 (d^{-1}). The differences between uptake parameters value might be due to differences in size and weight of the organisms used or the origin of the populations. Compared to the organisms of 32 mg used by Ashauer et al. (2010) (Ashauer et al., 2010), we used smaller organisms (7 mg) with larger surface-to-volume ration, potentially explaining a higher uptake rate. The difference in uptake rate constants of IMI between *C. dipterum* (2.96 $L \cdot kg_{ww}^{-1} \cdot d^{-1}$) and *G. pulex* (5.21 $L \cdot kg_{ww}^{-1} \cdot d^{-1}$) cannot be explained by length and weight (Figure S2 and Figure S3), as usually the smaller individuals accumulate more imidacloprid on a weight-specific basis than larger individuals.

After transfer of the contaminated organisms into clean water, internal IMI-concentrations decreased both in *C. dipterum* and *G. pulex*, showing that the chemical was being eliminated, metabolised or desorbed. The difference in elimination rates between the two species could attribute to the observed difference in sensitivity, as the sensitive *C. dipterum* (0.04 d^{-1}) eliminated slower than *G. pulex* (0.12 d^{-1}). As a result, the BCF_k of IMI estimated by the one-compartment TK model for *C. dipterum* was 1.58 times higher than that of *G. pulex* (Table 2). This might be partially explained by the fact that the elimination rate of IMI for *C. dipterum* was three times lower than for *G. pulex*, resulting in a three times longer persistence in the organisms (elimination half-life was 16.5 day for *C. dipterum* and 5.18 day for *G. pulex*).

The BCF of IMI-ole for *C. dipterum* was relatively high (8.00E+05 $L \bullet kg^{-1}$) compared to IMI (70.11 $L \bullet kg^{-1}$) as elimination rate of IMI-ole was very low (2.10E-06 d^{-1}), whereas the BCFk of IMI-ole was similar to IMI for *G. pulex*. The uptake difference of IMI and IMI-ole may be explained by their chemical properties as the lower Kow of IMI-ole compared to IMI (Table-S1), may result in a lower uptake rate. The elimination difference may result from the receptor affinities difference, since IMI-ole showed a slightly greater affinity to nAChRs than IMI (Casida, 2011; Nauen et al., 2001). Moreover, when exposed to IMI only, the metabolite IMI-ole was also detected in both taxa, indicating biotransformation of IMI into IMI-ole, which will be shown below.

3.2.2. Biotransformation TK model approach of IMI

Under IMI exposure, we detected both the parent compound and also the metabolite, IMI-ole (Fig. 1 A2, B2). IMI-ole was the only metabolite detected amongst the four metabolites we tested. IMI-ole was even still being formed during the elimination phase in both organisms (Fig. 1). The generation ratio of IMI-ole in *C. dipterum* was higher than in *G. pulex*, being 9.6% and 6.5%, respectively, at day 5 (see *SI.xlsx*).

Our result did not contradict the findings of Ashauer et al. (2010) (Ashauer et al., 2010), who did not find any metabolites in *G. pulex* after 1 day of IMI exposure, as in our tests the generation of metabolites in *G. pulex* was only detected after 2 days. The metabolization may have started at day 1 but was only present in measurable amounts on day 2. In the two model approaches (biotransformation TK model approach and the regular TK model approach), the k_u (k_{up} in eq. 1 and $b\_k_{u\_total}$ in eq. 4), in theory, should be the same for both model approaches, but was slightly different due to the model calibration. In the biotransformation model, the $b\_k_{e\_total}$ of the parent compound was divided into two

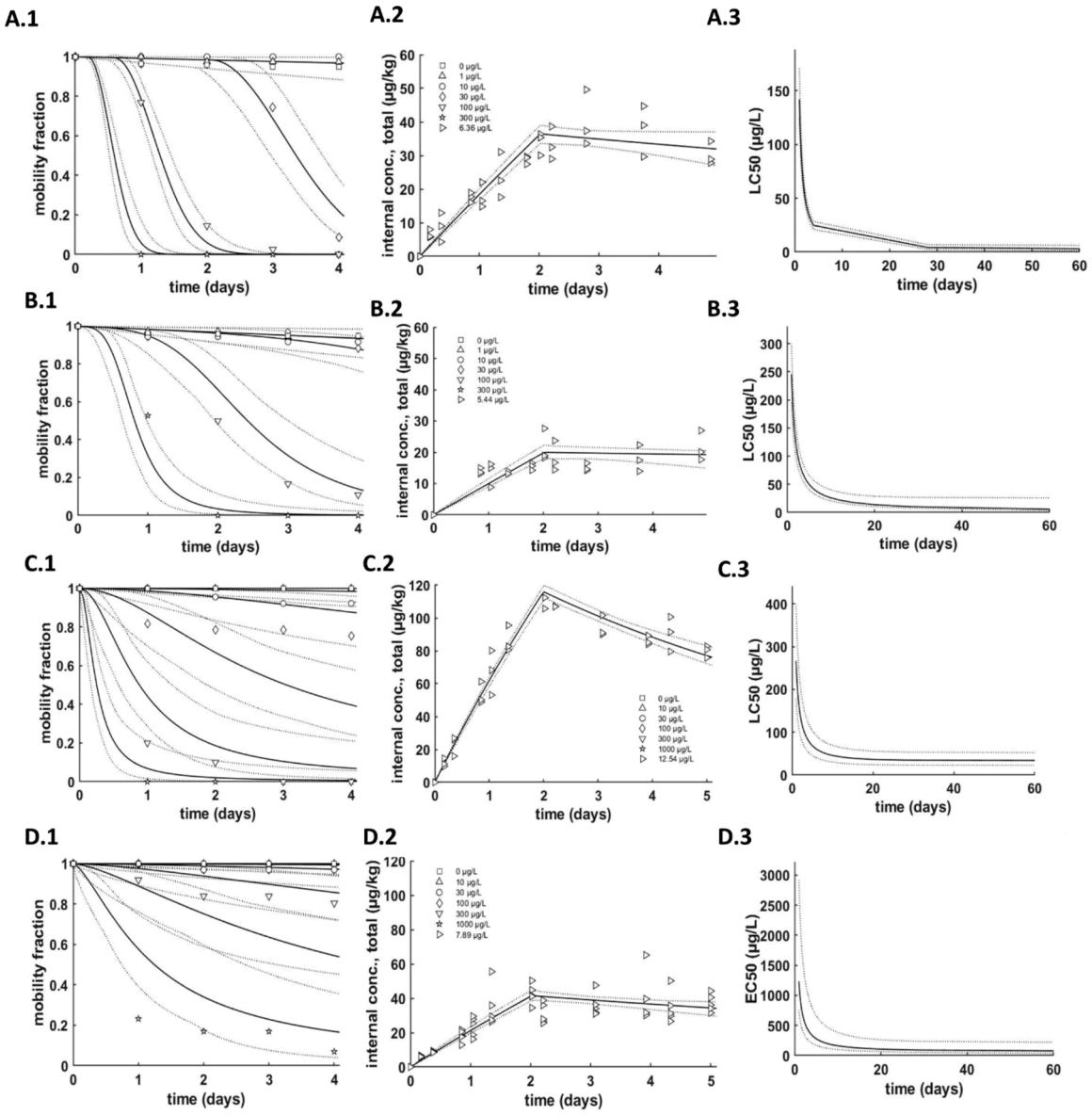

Fig. 2. The best-fitting GUTS model fitted on the data of IMI (A) and IMI -ole (B) for C. dipterum and of IMI (C) and IMI-ole (D) for G. pulex. The panels from left to right show (1) the mobility fraction (2) the internal concentration of IMI and (3) the predicted EC_{50} over time, respectively.

parameters, i.e. $b\_k_{bio\_parent}$ which is the biotransformation rate of IMI to IMI-ole and $b\_k_{e\_parent}$ which includes the elimination of IMI and the biotransformation rate of IMI to other potential metabolites than IMI-ole (eq. 4). For *G. pulex*, the elimination rate of IMI in both model approaches was close, while a difference factor of 2 was estimated for *C. dipterum*, although the confidence interval was overlapping. The BCFk values of IMI for each species were close for the two model approaches (Table 2).

The biotransformation model provided more details of the elimination pathway of IMI. In *C. dipterum* IMI was mainly depurated by biotransformation to IMI-ole (k_{bio} = 0.088 d^{-1}), which contributed 100% to the total elimination of IMI as the k_e of IMI or its transformation to other metabolites than IMI-ole was only 1.49E^{-10} d^{-1} (Table 2). In *G. pulex*, the k_{bio} of IMI was only 0.053 d^{-1} and biotransformation contributed only 41% to its depuration, as the k_e of IMI or its transformation to other metabolites than IMI was 0.076 d^{-1} (Table 2). Moreover, the estimated elimination rate of the metabolite, IMI-ole, by the biotransformation TK approach was 0 d^{-1} for both species. The very slow elimination of IMI-ole in *C. dipterum* was expected as it was also very slow when estimated by the one compartment TK model of IMI-ole exposure (Table 2, 2.10E^{-06} d^{-1}). When *G. pulex* is exposed to IMI-ole via the water phase, IMI-ole is eliminated quicker (k_{ep} = 0.07 d^{-1}; Table 2,) than when IMI-ole is formed as a metabolite ($k_{e\_metabolite}$ = 0 d^{-1}; Table 2), possibly because it is generated at the target sites and then binds tightly to them. To further confirm this internalising process, receptor binding assays need to be performed for aquatic invertebrates, as has been done for bees (Nauen et al., 2001).

The differences between the calculated BCF_k of IMI for both species alone could not explain the EC50 difference between them, as the results showed a 4.5 times difference with regard to 96h-EC50s and a 1.6 times difference with regard to BCFs. Hence, the biotransformation product also likely contributed to the toxicity differences of IMI-exposures between these two taxa. The higher toxicity of IMI to *C. dipterum* may be due to the lack of elimination of the toxic metabolite IMI-ole from the test organism (Table 1 and Fig. 2B.2). *G. pulex* also biotransformed IMI to IMI-ole, but to a lower extent than *C. dipterum* which results in a lower overall exposure (Table 2). Likewise, Suchail et al. (2001) suggested that for bees, imidacloprid seems to be a long-acting compound due to the

Table 2
The TK parameters of IMI and IMI-ole in C. dipterum and G. pulex as estimated by the simple TK model and the TK model that includes biotransformation (n.c represents not calculated).

Species	Model type	Exposure chemical	Water concentration (μg/L)	Measured chemical	Parameter k_u ($L \cdot kgww^{-1} \cdot d^{-1}$) k_e (d^{-1}) $b\_k_{bio\_parent}$ (d^{-1})	Value	95% CI	BCF $_k$ (L/kg)	$t_{1/2}$ (days)	R^2
C. dipterum	TK	IMI	6.36	IMI	k_{up}	2.96	2.62 - 3.33	70.11	16.44	0.92
					k_{ep}	0.04	0.00034 - 0.11			
		IMI-ole	5.44	IMI-ole	k_{up}	1.68	1.43- 1.94	8.00E+05	3.30E+05	0.81
					k_{ep}	2.10E-06	1.667e-08 - 0.1045			
	Bio-TK	IMI	6.36	IMI	$b\_k_{u\_total}$	3.18	2.973 - 3.381	36.14	7.88	0.92
					$b\_k_{e\_total}$	0.088	0.077–0.12			
					$b\_k_{e\_parent}$	1.49e-10	0 - 0.023			
					$b\_k_{bio\_parent}$	0.088	0.077 - 0.10			
				IMI-ole	$k_{e\_metabolite}$	0	0 - 0.0097	n.c	n.c	0.90
G. pulex	TK	IMI	12.54	IMI	k_{up}	5.21	4.87 - 5.54	44.41	5.91	0.98
					k_{ep}	0.12	0.11 - 0.16			
		IMI-ole	7.89	IMI-OLE	k_{up}	2.79	2.47 - 3.12	41.69	10.38	0.9
					k_{ep}	0.07	0.014 - 0.12			
	Bio-TK	IMI	12.54	IMI	$b\_k_{u\_total}$	5.13	4.66 - 5.77	39.59	5.15	0.98
					$b\_k_{e\_total}$	0.13	0.080–0.136			
					$b\_k_{e\_parent}$	0.076	0.034 - 0.13			
					$b\_k_{bio\_parent}$	0.053	0.046- 0.060			
				IMI-ole	$k_{e\_metabolite}$	0	0 - 0.057	n.c	n.c	0.98

generation of the metabolite IMI-ole.

Our results also indicate that a one compartment TK model was not informative enough to fully explain the difference in toxicity of IMI. This is because the metabolite IMI-ole was formed within the organisms and displayed similar toxicity as IMI, but had different elimination rates between the species. Hence, one needs to consider the toxicity and toxicokinetics of the biotransformation component as well, which is in agreement with other studies emphasizing the importance of biotransformation in toxicity process (Fu et al., 2018; Kretschmann et al., 2011).

3.2.3. *Prediction of long term effect of each compound by the GUTS model*

We integrated the results of the toxicity and TK experiments performed with IMI and IMI-ole and for both species in the GUTS model to predict the long term effect of each compound as described by (Jager et al., 2017). For IMI we compared the prediction with experimental 28 days toxicity data gained from (Roessink et al., 2013; Van den Brink et al., 2016). Our results showed that the full GUTS model provided a closer prediction of the observed chronic toxicity of IMI than the reduced model (Table S7). Further, we found that the results using the IT mechanism fitted the chronic data better than the SD mechanism, based on the smaller AIC and the higher goodness-of-fit measures (Table S8). Except for the toxicity of IMI to *C. dipterum*, which fitted better to the SD mechanism. Our result of *C. dipterum* with IMI was consistent with (Focks et al., 2018), as they also found that the SD mechanism explained better the toxicity of IMI to a variety of aquatic organisms. The result of *G. pulex* was consistent with Nyman et al. (2013), which found that the IT assumption fitted better in terms of the effect of IMI to *G. pulex* (Nyman et al., 2013), indicating that immediate effect occurs when the internal concentration in an individual exceeds its threshold.

The prediction of the 28d-EC_{50} of IMI to *G. pulex* 34.7 (22.9 - 53.4) μg/L was close to the chronic experiment results (Roessink et al., 2013) of 15.4 (9.80 - 24.1) μg/L; whereas the prediction of 28d-EC_{50} of IMI to a winter population of *C. dipterum* (4.3 μg/L, 2.4–6.9 μg/L) was higher than the experimental results (0.68 μg/L, 0.45 - 1.0 μg/L) (Van den Brink et al., 2016). The possible reason could be they used smaller organism (0.38 ± 0.05 cm) than in our toxicity and TK experiments (0.50 ± 0.07 cm) or more likely due to the biotransformation process in *C. dipterum* and the generation of a toxic and hardly eliminated metabolite, IMI-ole, which was not included in our model prediction. The discrepancy also emphasized the importance of incorporating active metabolites in further studies.

The prediction of the 28d-EC_{50} of IMI-ole was 10.4 (7.59–26.9) μg/L for *C. dipterum* and 135 (54.0–237) μg/L for *G. pulex*. Recently, Wan, et al. (2020) detected IMI-ole in filtered source water at concentrations 0.14 ng/L (Wan et al., 2020). However, we do not know much about the aquatic environmental occurrence and concentrations of IMI-ole, although some related studies can support the existence of IMI-ole in surface water. For example, imidacloprid and its metabolites may move into the water column through leaf degradation, since IMI, IMI-ole, and 5-OH-IMI have been detected in hemlock foliage tissue (Benton et al., 2015; Coots et al., 2013). A recent study found that although no IMI-ole could be detected in the water samples from headwater streams adjacent to hemlock stands treated with imidacloprid, they detected the bioaccumulation of IMI-ole (concentration of imidacloprid-olefin in the one sample was 28.9 ng/g) (Crayton et al., 2020). In combination with our results and current literature, the environmental water concentration of IMI-ole is low and may be relevant for the overall effects of IMI because IMI-ole is only formed by biotransformation by organisms and its elimination may be slow.

3.3. *Bioconcentration of IMI and the generation of its metabolites*

The toxicity of IMI to *C. dipterum* in this experiment was higher than expected based on the acute toxicity test results (Table 1 and Fig. 4). This was due to the use of smaller organisms in this experiment compared to previous acute toxicity tests (Fig. 4 and Figure S3). For *G. pulex*, half of the organisms died on day 4 when exposed to 1200 μg/L, while all organisms were immobile (Fig. 4). This was consistent with the results from the toxicity tests, and the sizes of *G. pulex* used in these two experiments were also similar (Fig. 4 and Figure S3). As all *C. dipterum* died on day 4, we only could get the internal concentration results in dead organisms (Fig. 3A, B), hence no comparison of the results for corpses, dead, immobile and mobile *C. dipterum* can be made. For *G. pulex*, the comparison amongst organisms of different status was usable (Fig. 3C, D, E, F).

3.3.1. *The passive absorption and active bioconcentration comparison*

This experiment aimed to estimate the contribution of passive absorption of IMI by the deceased organisms, in order to gain knowledge to what extend the physical absorption can account for the bioconcentration. The BCR of IMI for *C. dipterum* in the 120 μg/L treatments and *G. pulex* in 240 μg/L and 1200 μg/L treatments on the first day

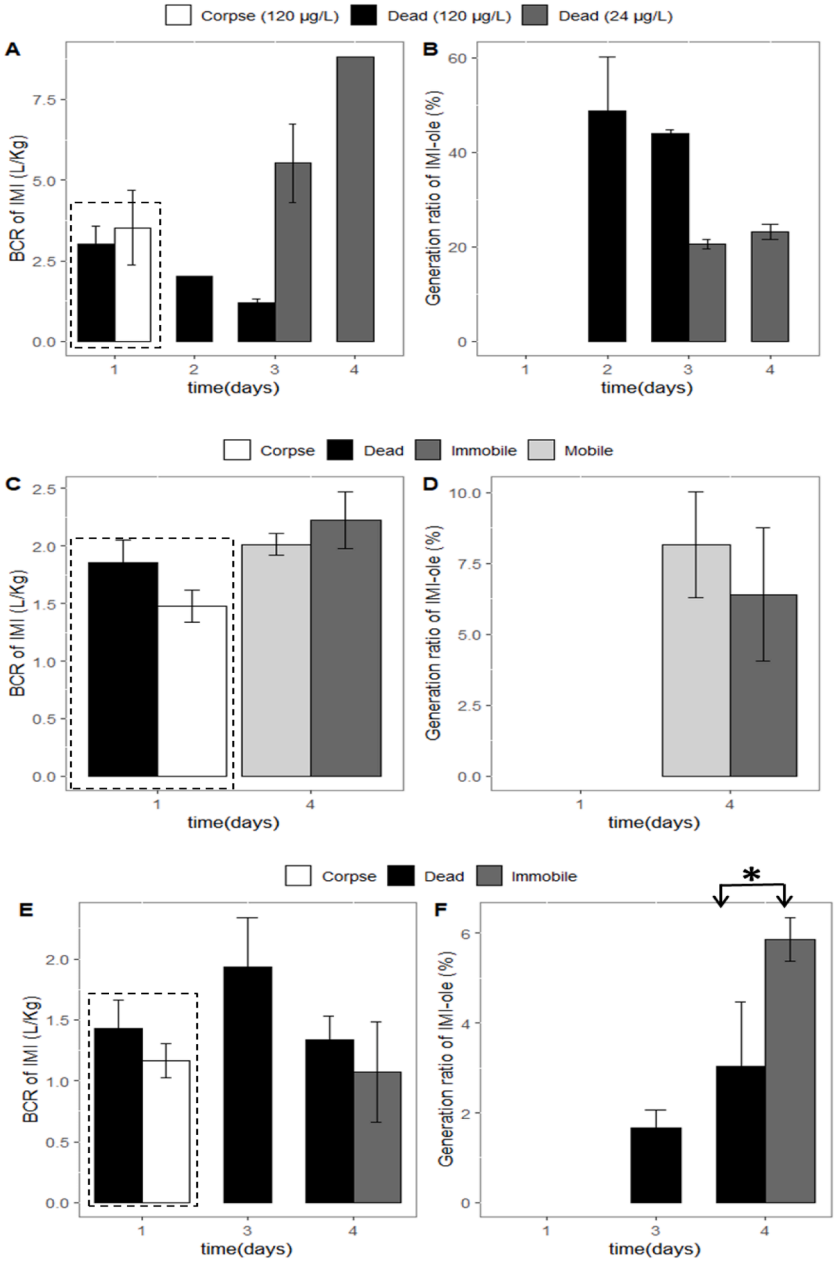

Fig. 3. The BCR (bioconcentration ratio) of IMI and generation ratio (in%) of IMI-olefin in C. dipterum are shown in panel A and B, respectively while those for G. pulex of the 240 µg/L treatment are shown in panel C, D and those for G. pulex of the 1200 µg/L treatment are shown in panel E and F, respectively. The dotted frame in A, C,E represents the comparison of passive absorption by deceased organisms (corpses) and the active and passive bioconcentration by organisms which died within day one. Corpse represented deceased organisms by freezing to evaluate the passive absorption of IMI, Dead represented organism who died during the experiment, immobile represented immobilized organisms during the experiment. * denotes a significant difference. Error bars represent standard error of the mean.

showed no difference between the passive absorption by deceased organisms (corpses) and the passive and active uptake by organisms which died within day 1 in the test (Fig. 3A, C, E dotted frame). But the BCR of IMI for the deceased *C. dipterum* was higher than for *G. pulex* (Fig. 3A, C, E dotted frame), which might be due to their length and weight (Figure S2 and Figure S3), as smaller individuals accumulate more imidacloprid on a weight-specific basis than larger individuals. Smaller organisms accumulated more in case of passive absorption (Fig. 3A, C, E dotted frame part) and this relationship was also found by Rubach et al. (2010) for another insecticide, chlorpyrifos (Rubach et al., 2010). Although both the exact time of death and the dynamics of active bioconcentration within 1 day by the organisms were unknown, the comparison indicated that passive absorption should not be neglected. In other words, a considerable amount of IMI adsorbed to the animal's surface or exoskeleton passively. Miller et al. (2016), who analysed the exoskeleton of gammarids that moulted during the exposure period found that the percentage of the total body residue adsorbed to the exoskeleton was between 3 and 24% for five pharmaceuticals (Miller et al., 2016). In order to perform further research and compensate for the analytical limitation of our method,some analysis methods, such as mass spectrometry imaging (Ewere et al., 2019) can provide more information on the distribution of compounds in or on the organisms.

3.3.2. *The BCR of IMI and the generation ratio of its metabolites in organisms with different status*

The BCR of IMI was higher in *C. dipterum* than in *G. pulex* (Fig. 3A, C, E). In addition, in both species, IMI-ole was the only metabolite detected in the body after day 1 for *C. dipterum* and after 2 days for *G. pulex*, while *C. dipterum* generated more IMI-ole than *G. pulex* (Fig. 3B, D, F), which agrees with the previous TK results (Fig. 2). The BCR of IMI in the dead

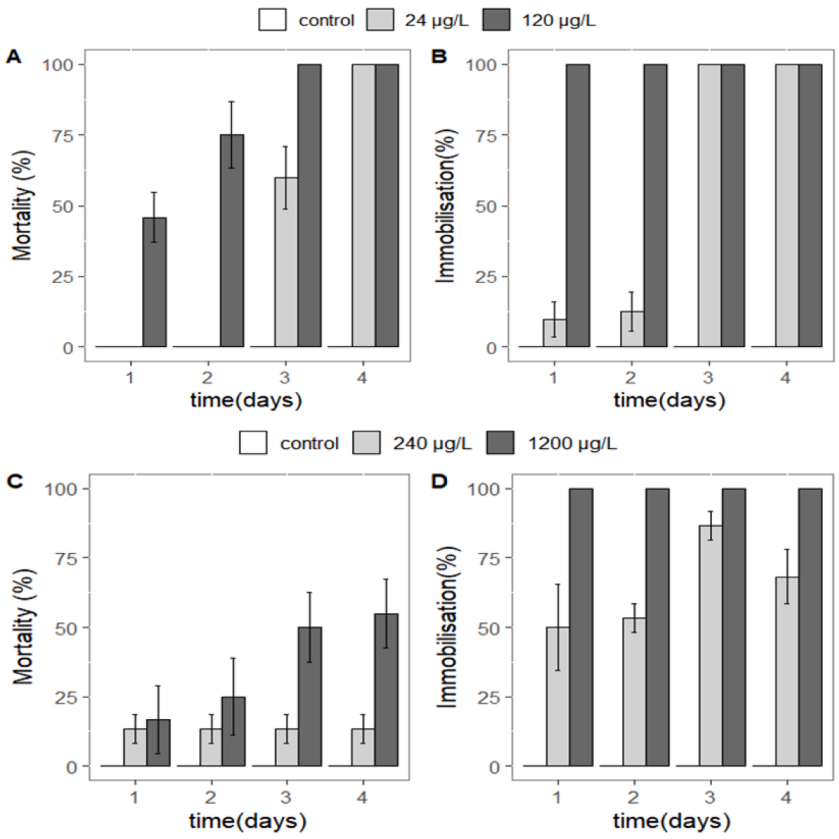

Fig. 4. The mortality and immobilisation of C. dipterum (panel A and B), mortality and immobilisation of G. pulex (panel C and D) in the different concentration treatments ($n = 6$). Error bars represent standard error of the mean.

C. dipterum in the 120 μg/L treatment decreased over time (Fig. 3A, $p = 0.017$). This may be due to the generation of unknown metabolites because the ratio of IMI-ole did not change between day 2 and day 3 (Fig. 3B). The BCR of IMI in the dead *G. pulex* in the 1200 μg/L treatment did not change over time (Fig. 3E). The generation ratio of IMI-ole was significantly higher in immobile than in dead organisms (Fig. 3F, $p = 0.034$,) on day 4, as a result of the termination of the biotransformation of IMI when organisms died. As for the comparison amongst another

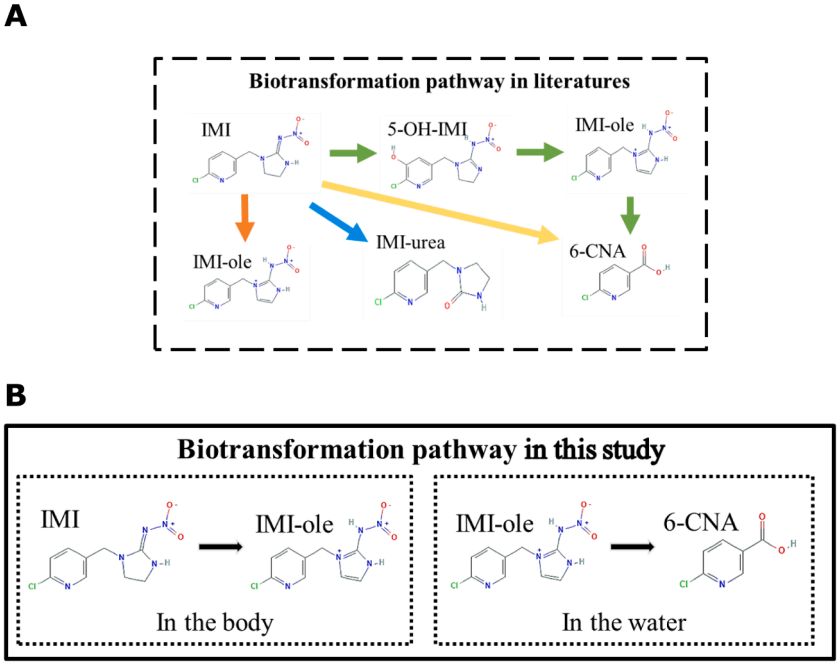

Fig. 5. The biotransformation pathway of IMI deduced from the literature (A), different colours represent different pathways, and from the results of this study (B).

status, there were no significant differences.

The high generation ratio of the toxic metabolite IMI-ole, especially for *C. dipeturm* (49%) was in line with another study which found IMI-ole concentrations to be 3.4 fold higher than that of IMI after a 46-day dietary exposure study with bees (Erban et al., 2019). Our findings are also consistent with other studies which found that IMI-ole was the major metabolite after exposing IMI to termites (Tomalski et al., 2010), lizards (Wang et al., 2018) and bees (Erban et al., 2019). In our study, compared to *G. pulex,* the more sensitive mayfly had a higher internal concentration of IMI and its toxic metabolite, IMI-ole. This result indicates that the generation of the metabolite IMI-ole, which was persistent in the body (see Section 3.2 TK results), contributes to the overall IMI toxicity.

We summarised four possible metabolic pathways based on published information on the biotransformation of IMI in organisms (Fig. 5A). These comprised 1) the pathway where IMI was transformed to 5-OH-IMI, then to IMI-ole (Tomalski et al., 2010), and might be further transformed to 6-CNA (Nishiwaki et al., 2004); 2) the pathway where IMI was transformed to IMI-urea, 3) the pathway where IMI was transformed immediately into 6-CNA (Fusetto et al., 2017); and 4) where IMI was transformed to IMI-ole (Fusetto et al., 2017) (Fig. 5A). Integrating all our toxicity, internal kinetic and biotransformation results, we conclude that amongst the four selected metabolites (IMI-ole, 5-OH-IMI, IMI-urea, 6-CNA), IMI-ole was generated within both organisms once they were exposed to IMI after 1 or 2 days; 6-CNA was detected in the water when both organisms were exposed to IMI-ole (Fig. 5B). Suchail et al. (2004) summarised that IMI generates 6-CNA through a putative metabolite, 6-chloropicolyl alcohol (Suchail et al., 2004a). We hypothesise that IMI was biotransformed to IMI-ole, after which IMI-ole may be biotransformed further to 6-CNA. To our knowledge, the formation of 6-CNA from IMI-ole has not been reported before for invertebrates.

4. Conclusions

The biotransformation product IMI-ole showed similar toxicity to *C. dipterum* and somewhat lower toxicity to *G. pulex* compared to the parent compound IMI. Whilst IMI was biotransformed to IMI-ole in both species at considerable rates, it showed almost no elimination from *C. dipterum* and a slow one from *G. pulex*. During exposure to IMI, the bioactive metabolite IMI-ole could be detected after one-day of exposure and reach relatively high concentrations in *C. dipterum* compared to *G. pulex* contributing to the toxicity of IMI. Our results suggest that IMI-ole may gradually be generated, especially in *C. dipterum,* and not being eliminated by this species. Hence IMI-ole may be responsible for the chronic toxicity of IMI in *C. dipterum* with increasing exposure times. It also demonstrates that the sensitivity difference between invertebrates depends not only on the bioaccumulation of the parent compound, but also on its biotransformation and elimination.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105837.

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