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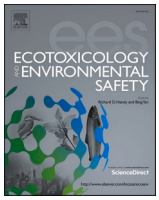

Comparing the acute and chronic toxicity of flupyradifurone and imidacloprid to non-target aquatic arthropod species

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ABSTRACT

Flupyradifurone (FPF) is a new type of butenolide insecticide. It was launched on the market in 2015 and is considered an alternative to the widely used neonicotinoids, like imidacloprid (IMI), some of which are banned from outdoor use in the European Union. FPF is claimed to be safe for bees, but its safety for aquatic organisms is unknown. Its high water solubility, persistence in the environment, and potential large-scale use make it urgent to evaluate possible impacts on aquatic systems. The current study assessed the acute and chronic toxicity of FPF for aquatic arthropod species and compared these results with those of imidacloprid. Besides, toxicokinetics and toxicokinetic-toxicodynamic models were used to understand the mechanisms of the toxicity of FPF. The present study results showed that organisms take up FPF slower than IMI and eliminate it faster. In addition, the hazardous concentration 5th percentiles (HC_{05}) value of FPF derived from a species sensitivity distribution (SSD) based on acute toxicity was found to be 0.052 μmol/L (corresponding to 15 μg/L), which was 37 times higher than IMI (0.0014 μmol/L, corresponding to 0.36 μg/L). The chronic 28 days EC_{10} of FPF for *Cloeon dipterum* and *Gammarus pulex* were 7.5 μg/L and 2.9 μg/L, respectively. For *G. pulex*, after 28 days of exposure, the no observed effect concentration (NOEC) of FPF for food consumption was 0.3 μg/L. A toxicokinetic-toxicodynamic (TKTD) model parameterised on the acute toxicity data well predicted the observed chronic effects of FPF on *G. pulex*, indicating that toxicity mechanisms of FPF did not change with prolonged exposure time, which is not the case for IMI.

1. Introduction

Neonicotinoids are among the most used pesticides, and they are currently registered for hundreds of field crops in over 120 countries, accounting for one-third of the pesticide market share (Jeschke and Nauen, 2008; Jeschke et al., 2011; Simon-Delso et al., 2015). However, the adverse effects of neonicotinoids on bees and non-target aquatic invertebrates have been raised with increasing concern (Morrissey et al., 2015; Jactel et al., 2019). In 2018, three neonicotinoids, imidacloprid (IMI) (European Commission, 2018a), clothianidin (European Commission, 2018b) and thiamethoxam (European Commission, 2018c), were banned for outdoor use in Europe because of their risks to pollinators. One of the most important alternatives to neonicotinoids is flupyradifurone (FPF), a new butenolide pesticide (Giorio et al., 2017). FPF was first commercially available in Honduras and Guatemala in 2014 (Nauen et al., 2015a) and has since become available for use on a wide range of crops in Canada (PMRA, 2015), the United States (EPA, 2015), China (Zhong et al., 2021) and Europe (EFSA, 2016). It acts as an agonist on insect nicotinic acetylcholine receptor (nAChR), similar to neonicotinoids (Jeschke et al., 2015b), while it has a different pharmacophore system as a new bioactive scaffold (Nauen et al., 2015b). FPF is used for a wide range of crops as a foliar spray, soil drench, and seed treatment, targeting sucking pests such as aphids, hoppers, and whiteflies (Bayer, 2012). According to a new economic assessment, FPF currently is one of the most commonly used imidacloprid substitutes in Californian agriculture (Goodhue et al., 2020). Research shows that FPF seems comparatively safer for honeybees and bumblebees than IMI (Campbell et al., 2016).

However, the knowledge of the safety of FPF to freshwater invertebrates is limited. Considering the chemical characteristics of FPF, i.

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e., high solubility in water, low volatility and high half-life values in water and/or soils (Bayer, 2012; Nauen et al., 2015a), FPF will be persistent in the environment and has a high potential to enter freshwater ecosystems through runoff, erosion, and leaching (Carleton, 2014). So far, there are not many studies on environmental concentrations of FPF, but one study found that the highest measured environmental concentration of FPF is 0.16 μg/L in a watershed of the Great Lakes basin (Metcalfe et al., 2019). To the best of our knowledge, toxicity data of FPF is alone available for three aquatic arthropod species: *Hexagenia* spp. (Bartlett et al., 2018), *Chironomus dilutus* (Maloney et al., 2020) and *Hyalella azteca* (Bartlett et al., 2019). Notably, data for Daphnia were excluded because FPF is, like IMI, not toxic to cladocerans (Lewis et al., 2016). These available studies focused on lethal endpoints, such as mortality and immobility, conventionally assessed in 2 or 4 days acute and 28 days chronic tests. Acute and chronic toxicity tests have been performed with aquatic invertebrates extensively for neonicotinoids, especially for IMI (Roessink et al., 2013; Morrissey et al., 2015). Besides lethality-related endpoints like mortality and immobilisation, insecticides may also affect sublethal endpoints, such as feeding rate (Nyman et al., 2013), growth, reproduction, and emergence (De França et al., 2017). In the case of IMI, the sublethal effect concentration was found to be several orders of magnitude lower than the lethal concentration, and importantly, the sublethal effect on individuals may result in a profound impact on populations (Rico et al., 2018; Van de Perre et al., 2021). Therefore, more studies on the effects of FPF on freshwater invertebrates focussing on the lethal and sublethal endpoints, are needed.

To understand the mechanism of the toxicity of pesticides, toxicokinetic-toxicodynamic (TKTD) models can be used (Ashauer and Escher, 2010). Specifically, models from the General Unified Threshold model for Survival (GUTS) can help to understand the mechanisms and characteristics of lethal effects. Recently, an EFSA (European Food Safety Authority) working group concluded that the GUTS models are fit for purpose of being used for the risk assessment of pesticides for aquatic organisms (EFSA PPR Panel (Panel on Plant Protection Products and their Residues) et al., 2018). GUTS modelling was also successfully used to predict survival over time of aquatic invertebrates exposed to time-variable exposure patterns of IMI (Focks et al., 2018).

To assess the toxicity of FPF for aquatic arthropods, we selected three species, the ephemeropteran *Cloeon dipterum*, the amphipod *Gammarus pulex,* and the isopod *Asellus aquaticus*. They are widely distributed invertebrates in Europe and play an important role in freshwater ecosystems (Hynes, 1970). Besides, because of their high abundance, high reproduction rate, and comparably short life cycle, they are suitable organisms for laboratory toxicity tests (Williams et al., 1984; McCahon and Pascoe, 1988; Johnson et al., 1993). Also, the insect species *C. dipterum* is one of the most sensitive species to IMI, while *G. pulex* and *A. aquaticus* are macrocrustaceans and are intermediately sensitive to IMI (Roessink et al., 2013). Using these three species, we aimed to compare the toxicity of IMI and FPF for species belonging to different taxonomic groups.

The objectives of this study were to evaluate the effects of FPF on lethal and sublethal endpoints resulting from acute and chronic exposure and to use GUTS modelling to test for consistency between acute and chronic toxicity of FPF. For these objectives, we performed a 4-day acute toxicity test with three species, *C. dipterum*, *G. pulex,* and *A. aquaticus*, and consecutive toxicokinetic experiments with the same species. From that, uptake and elimination rates of FPF were derived and compared to literature values available for IMI. Previous acute toxicity tests performed with arthropods obtained from the literature were integrated with our results to generate an acute species sensitivity distribution (SSD) for FPF and IMI. Effects on mortality, immobility, size, emergence, food consumption and internal concentrations were assessed in the chronic test of FPF with *C. dipterum* and *G. pulex*.

2. Materials and methods

2.1. Chemicals and test organisms

Flupyradifurone (CAS: 951659-40-8) and the commercially available metabolite, 6-chloronicotinic acid (6-CNA; CAS: 5326-23-8), were used in the experiments. The stock solutions of FPF (10 and 1 μg/mL) were dissolved into MiliQ water. Imidacloprid-d4 (IMI-d4; CAS: 1015855-75-0) was used as an internal standard during the analytical measurements of all organism samples. The stock solutions of IMI-d4 (200 μg/mL) were dissolved into 2 % acetone (v: v) to ensure that the compound was fully dissolved. The FPF stock solution was stored at 4 °C in the dark when it was not used. A fresh stock solution was prepared for each test.

Three species, *C. dipterum*, *G. pulex* and *A. aquaticus*, 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 N, 5.748697E), while *A. aquaticus* was collected from the campus of Wageningen University and Research (a pond with the coordinates 51.986859 N, 5.668837E). 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 with fish food for *C. dipterum* and leached *Populus* leaves for *G. pulex* and *A. aquaticus* (Roessink et al., 2013). All containers containing the test organisms were placed in a water bath maintained at 18 ± 1 °C with a light: dark regime of 12:12 h. The light in our setup did not contain ultraviolet light to prevent photodegradation of FPF.

In order to confirm the absence of all tested analytes in the collected water and organisms samples, we analysed the water and organisms samples by LC/MS-MS before the exposure. For the details of chemical analysis, see Section 2.5.

2.2. Acute toxicity experiments

The acute toxicity of FPF was assessed by a 4-day standard acute toxicity test to estimate the 96 h EC_{50} and LC_{50} for *C. dipterum*, *A. aquaticus* and *G. pulex* (Roessink et al., 2013). In detail, separate tests were performed for each species. For *C. dipterum,* each replicate consisted of 16 mayflies placed in a glass jar containing 1 L groundwater obtained from the Sinderhoeve experimental station. This volume was dosed with FPF to reach exposure concentrations of 0, 1, 3, 10, 30, 100 and 300 μg/L. For the tests with *G. pulex* and *A. aquatics*, each replicate consisted of 16 individuals (*G. pulex)* or 10 individuals (*A. aquaticus)* in 1 L groundwater, after which the volume was dosed to reach concentrations of 0, 3, 10, 30, 100, 300 and 1000 μg/L. These experimental concentrations were achieved by adding an appropriate portion of the stock solution to the test system, and the selection of the concentration was based on the results of previous IMI and FPF toxicity studies with aquatic arthropods (Roessink et al., 2013; Maloney et al., 2020). These tests were performed in May 2020, using a summer generation of all species. Experiments were performed with three replicates per treatment level, while five replicates were used for controls. The test systems were not aerated during the experiments to minimise water evaporation. In the experiments with *G. pulex* and *A. aquaticus,* a piece of stainless steel mesh was added to serve as a substrate for organisms and reduce cannibalistic behaviour. Organisms were checked every day, and the status (dead, immobile or mobile) of each individual was assessed according to Roessink et al. (2013). Dead organisms and moults were removed daily.

Daily, 1 mL of water was taken to verify the exposure concentration of FPF by 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 during the acclimation period. Dissolved oxygen, pH, electrical conductivity and temperature were measured at the start and end of the test in the control group and in the highest treatment only. The dissolved

oxygen content was acceptable at the end of the experiment, with a minimum value of 8.7 mg/L. These results are provided in the raw dataset (Huang et al., 2022a).

2.3. Toxicokinetic tests

At the end of the acute toxicity test (day 4), less than 10 % of the individuals were affected in the 10 μg/L treatment for all three species. With the remaining organisms of these treatments, a toxicokinetic test was conducted. In detail, after 4 days of exposure, for the acute tests with *C. dipterum* and *G. pulex*, 8 individuals were randomly selected from each replicate separately, rinsed for 30 s with clean MiliQ water, and transferred to 1 L clean groundwater to assess the elimination of FPF after 2 days. The remaining 8 individuals were cleaned and stored at −20 °C for further chemical analysis and were used to determine the uptake after 4 days. The test with *A. aquaticus* used 5 individuals for the uptake and 5 individuals for the elimination phase. At the end of the elimination phase, all alive organisms were collected from the three replicates, washed with MillQ water for 30 s, and stored at −20 °C for further chemical analysis.

The concentration of FPF was measured in both organisms and water samples during the uptake, and elimination phase for each replicate separately. For quality control of the experimental setup, three replicates containing organisms but no chemicals (from the previous control groups) were also kept during the elimination phase to evaluate the status of the organisms.

2.4. Chronic experiment

As *A. aquaticus* was the least sensitive species in the acute experiments, the chronic tests were only performed with *C. dipterum* and *G. pulex* in September and November 2020. The experimental conditions and spiking procedure of FPF were the same as in the acute tests. The volume was dosed to reach final exposure concentrations of 0, 0.1, 0.3, 1, 3, 10, 30 μg/L for *C. dipterum* and 0, 0.3, 1, 3, 10, 30 μg/L for *G. pulex*. These experimental concentrations were achieved by adding an appropriate portion of the stock solution to the test system, and the selection of the concentration was based on our previous FPF acute test results. Gentle aeration was provided, and the animals were fed with appropriate species-specific food, weekly for *C. dipterum* and biweekly for *G. pulex*. 1 mL of fish flask slurry, consisting of 6 g ground fish flask added to 40 mL MillQ water and homogenised by stirring with the magnet mixer, was added to each replicate for *C. dipterum*, and two pieces of discs of leached *Populus* leaves (3.2 cm radius) for *G. pulex*. The jars were completely refreshed with a new test medium and food every week for *C. dipterum* and every two weeks for *G. pulex,* and the living test animals were transferred with care to the new test system. Immobility and mortality were monitored every 2–3 days during the experiment, and the physicochemical water parameters were measured weekly. For *C. dipterum,* the emergence of individuals occurred mostly in the fourth week of the experiment. Emerged *C. dipterum* was counted as missing in the statistical analysis because, after emergence, it is no longer possible to determine whether the individual would have been affected or not.

The food consumption of *G. pulex* was measured every two weeks in this study. The pre-treated (leached into the water before use) (McGrath et al., 2007) *Populus* leaves were cut into circles with the same surface area (3.2 cm radius) using a cork borer and dried at 60 °C for at least 48 h. Two pieces of leaf discs were provided for every replicate. The dry weight of the leaves of each replicate was recorded before putting them into the jar. The dry leaves were added to each replicate for 3 days before adding the organisms and the chemical to allow the leaves to soak in the clean water. The leaves in the test jars were changed every two weeks together with the refreshment of the system. The weight of the remaining leaves was recorded after drying at 60 °C in the oven for at least 48 h. Apart from the jars with *G. pulex*, a blank food treatment without organisms was added. Two jars with only stainless-steel mesh and conditioned Populus leaves were installed to estimate the microbial degradation of the leaves. The food consumption of each replicate was calculated as the difference between the initial leaves weight and the remaining leaves' weight after the loss was corrected for microbial degradation.

At the beginning and during the system renewal process, *C. dipterum* was taken out to measure their length using a binocular with a camera (Olympus, U-TV0.5XC-3, Japan). At the beginning and the end of the experiment, a short video (less than 10 s) of all *G. pulex* individuals present in a replicate was taken. The video screenshots were extracted from the videos with Elmedia Video Player (7.17, Elmedia Software). The body length of *C. dipterum* was measured from the anterior margin of the mesothorax to the posterior end of the abdominal segment. The body length of *G. pulex* was measured from the anterior margin of the caput to the posterior end of the pleon (not including telson), along with the curved shape of the pereon (Fig. S1). Each organism was observed under the microscope with a reference object for scale. The programme ImageJ (1.53, National Institute of Health, USA) was used for image analysis and length measurements (SI Text S1).

After 28 days of exposure, all remaining organisms were washed with MillQ water for 30 s and stored at − 20 °C for further chemical analysis. The concentration of FPF was measured in both the organisms and the water.

2.5. Chemical analysis

In the toxicokinetic experiment (Section 2.3) and at the end of the chronic experiment (Section 2.4), the internal concentration of FPF was measured at each time point in each test. The remaining organisms from the same jar were pooled for the measurement, which resulted in 3 replicates for each concentration level. The methods were similar to those in (Huang et al., 2021). In detail, 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 obtain the dry weight of the 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 homogenised 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 centrifuged at 10,000 rpm for 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 which may remain in the filter. This filtrate, in turn, was centrifuged 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) using the protocol of imidacloprid, with small modifications (Huang et al., 2021). The injection volume of the samples was set at 10 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 an Agilent jet stream electrospray ionisation source (AJS-ESI) in positive mode. Nitrogen was used both as nebuliser 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 the multiple reaction monitoring (MRM) using two transitions per compound.

The MS/MS transitions of all compounds are provided in Table S1.

Injected samples were quantified by peak area using the calibration curve constructed from the calibration standards included in the same sample sequence (Tables S2 and S3). Agilent Masshunter software (version 8.0) was used for instrument control and data acquisition. The extraction recoveries of FPF in the organisms, evaluated at two concentrations (a low and a high concentration) by spiking them into the clean organisms, were acceptable for all three species tested based on recovery and repeatability (Table S4). The limit of quantification (LOQ) was calculated based on the measurement of the analyst responses in the sample matrix corresponding to a signal-to-noise ratio (S/N) of 10:1. Determination of the S/N was performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified (Table S2–S4). More information on the measurement is presented in Text S2.

2.6. Data analysis

2.6.1. Species sensitivity distribution (SSD)

We used the Ecotox database (www.epa.gov/ecotox) to collect acute toxicity data for IMI and FPF of aquatic arthropods. A direct comparison between the IMI and the FPF toxicity was made based on the mortality or immobility toxicity values of the same six benthic arthropod invertebrate species (Table 1 and references therein). For this comparison, the toxicity values for IMI and the FPF were obtained from the same study or the same research group.

Species sensitive distributions (SSD) were generated based on all currently available data. All the criteria (test duration, etc.) for data selection were based on a previous study (Maltby et al., 2005). To be specific, the taxa of interest were aquatic Arthropoda. The selected endpoints were the median effect concentration (EC_{50}) regarding immobility of animals or the median lethal concentration (LC_{50}) when the EC_{50} value was not available. Effect concentration (ECx) is a better indicator of toxicity than the lethal concentration (LC_{50}) for IMI since most organisms in short-term acute toxicity tests are only immobilised but not dead, but they will die after prolonged exposure (Roessink et al., 2013; Raby et al., 2018). Only test results with a duration between 1 and 7 d were included. A genus-specific geometric mean was used when no specific species names were provided. For species with multiple entries in the database, we first distinguished them by the duration of experiment and then by study. Specifically, If they were from the same study, but on different days, we only selected the last day's value; if they were from different studies, we used the geometric mean of all values. The SSDs based on these EC_{50} or LC_{50} values were fitted using a log-normal distribution using maximum likelihood (Xu et al., 2015; EPA, 2016). To be noted, the values for Daphnia were excluded as both chemicals are not toxic to cladocerans (Morrissey et al., 2015; Li et al., 2021). In summary, we had data for 6 species (including 3 species from our study) to generate the acute SSD of FPF and 39 species for IMI. Furthermore, to compare the difference between the Crustacea and Insecta, subphyllum-specific SSDs were calculated for IMI (see SI Text S3).

2.6.2. Lethal concentrations and effect concentrations

Lethal concentrations (LCx) and effect concentrations (ECx), were determined for each observation time point by fitting the number of dead and affected organisms, respectively, to a 4-parameter log-logistic model (LL.4) with the drc package (Ritz and Streibig, 2005) in the open-source software R version 4.1.0 (Ritz et al., 2015).

$$f(x) = c + \frac{d - c}{1 + exp(b(log(x) - \log(e)))} \quad (1)$$

With *f(x)* is the fraction of affected or dead organisms, *b* is the slope around LC_{50} or EC_{50} (which is *e*), *c* denotes the control mortality, *x* is the water concentration, and *d* is the upper limit.

Mortality and immobility data fit the LL.4 model with the upper limit of 1 and are based on a binomial distribution.

2.6.3. Food consumption

Dry mass (DM, mg) of leaves consumed by *G. pulex* per jar (L_e) after two weeks was calculated as:

$$L_e = L_i - L_f - Lc. \quad (2)$$

Where $L_i$and L_f *are* the initial and final dry mass (mg) of leaves, and *Lc* is the average dry mass loss of the blank food treatment accounting for microbiological degradation.

To gain food consumption per organism, we divided the total amount of food consumed per jar by the numeric mean of the remaining organisms at each observation time (Eq. 3). The food consumption rate per organism (F_{total}) after two weeks was calculated as:

$$F_{total} = \frac{L_e}{average \quad (n1 + n2 + \ldots + nt)} \quad (3)$$

Where *n* is the numeric mean of remaining organisms at each observation time, day 1, 4, 7, 9, 11 and 14 for the first two weeks and day 14, 16, 18, 21, 23, 25 and 28 for the last two weeks.

2.6.4. Calibration of TKTD models and predictions of survival

The GUTS TKTD framework has been described earlier (Jager et al., 2011; Jager et al., 2017; Jager, 2021). The GUTS models were calibrated using MATLAB (2021b), using the BYOM modelling platform (www.debtox.info/byom.html). We used both the reduced and the full cases for the two death mechanisms (SD, IT). We started by fitting the reduced model (GUTS-RED) to the acute survival data (Section 2.2) alone. The use of GUTS-RED allows fitting a TKTD model in the absence of information on body residues.

Also, the full model (GUTS-FULL) was used for fitting survival and body-residue data together; more precisely acute survival data (Section 2.2) and the internal concentration measurements of toxicokinetic

Table 1
Comparison of effect concentrations (in two units, μg/L and μmol/L) causing 50 % mortality (LC_{50}) or immobility (EC_{50}) of imidacloprid (IMI) and flupyradifurone (FPF).

Species	Class	Life stage	Size/weight/age	endpoint category	IMI (μmol/L)	IMI (μg/L)	FPF (μmol/L)	FPF (μg/L)	Temp-erature (°C)	Reference
Cloeon dipterum	Insect	juveniles	5 mm big	EC_{50}	3.9E-03	1[b]	0.1[a]	42	18	(Roessink et al., 2013)
Gammarus pulex	Crustacean	juveniles	5 mm big	EC_{50}	0.1	18[b]	0.3[a]	94	18	and present study;
Asellus aquaticus	Crustacean	juveniles	5 mm big	EC_{50}	0.5	119[b]	0.5[a]	137	18	(Bartlett et al., 2019)
Hyalella azteca	Crustacean	juveniles	2–10 days old	LC_{50}	0.9	230	0.1	26	25	(Maloney et al., 2020)
Chironomus dilutus	Insect	larvae	6–7 days old	LC_{50}	2.7E-02	7	0.1	17	23	(Maloney et al., 2020)
Hexagenia spp.	Insect	larvae	5–8 mg weight	EC_{50}	3.9E-02	10	0.3	81	n.d	

Note: a= Data from the present study, b= Data from the same group
n.d = not mentioned.

(Section 2.3) were used. In addition, we compared the toxicity predictions based on the calibrated GUTS-RED and GUTS-FULL models with our FPF chronic test results to evaluate the possibility of extrapolating acute toxicity values to chronic toxicity values using a TKTD model. For *G. pulex*, 28-day chronic data were used. As *C. dipterum* emerged after 21 days, only the chronic survival data within 17 days was used for the prediction. The Normalised Root Mean Square Error (NRMSE) was used in our study to evaluate model performance (EFSA PPR Panel (Panel on Plant Protection Products and their Residues) et al., 2018). A further description of the GUTS framework is provided in the supporting information Text S4.

2.6.5. Statistical analysis

Significant differences between treatments and controls were assessed by using R (version 4.1.0). The assumptions of normality were evaluated with a Shapiro-Wilk test, and the assumption of equal variance was assessed using a Spearman rank correlation between the residuals and the dependent variable. If the assumptions of normality and equal variance were passed, a one-way analysis of variance (ANOVA) with $\alpha = 0.05$ and a post-hoc Tukey's test was conducted. If assumptions failed, a Kruskal-Wallis test, with $\alpha = 0.05$, and a post-hoc Dunn's test were used.

3. Results and discussion

In both acute and chronic experiments, the water concentration of FPF was stable and within 20 % deviation from the nominal concentration (Huang et al., 2022a). Thus, the nominal concentrations have been used to describe the treatment levels in the following results and discussions. However, in order to be more accurate and to be able to capture subtle differences, the average measured water concentration was used in the TK and GUTS modeling.

3.1. The accumulation, uptake and elimination of FPF

3.1.1. The accumulation of FPF after 28 days of exposure

After a chronic 28-day experiment, we measured the internal concentration of FPF in the remaining living organisms (Table 2). The bioconcentration factor (BCF) was calculated as we expected that the bioconcentration process would reach a steady state after 28 days of exposure because of the quick elimination time (t_{95}) of FPF (Table 3). The t_{95} is the time that it would take the organism to eliminate 95 % of the accumulated toxicant when returned to clean water, which is equivalent to the time to reach the steady state (Ashauer et al., 2010; Rubach et al., 2010). Because FPF is highly water-soluble, we estimated that the contribution of food uptake to the accumulation of FPF was low (Nauen et al., 2015a); thus, no accumulation via food intake was considered in the present study.

Overall, *C. dipterum* accumulated less FPF than *G. pulex* under the same exposure concentration (Table 2). For *C. dipterum,* the BCF values were similar for the different treatments (Table 2). However, for *G. pulex,* the BCF values decreased with increasing concentration levels.

Table 2
The bioconcentration factor (BCF) and internal concentration of flupyradifurone in two species after 28 days of exposure.

Species	exposure concentration (μg/L)	BCF (L/kg)	internal concentration (μg/kg) (mean value ± sd, n = 3)	
C. dipterum	1	0.71	0.71	±0.44
	3	2.26	6.79	±1.46
	10	1.47	14.7	±7.11
G. pulex	0.3	49.5	14.9	±2.32
	1	35.4	35.4	±6.71
	3	19.2	57.5	±2.07
	10	7.56	75.6	±6.75

Although we did not evaluate passive absorption in this study, the adsorption of FPF on the surface of the Gammarus exoskeleton could explain this. A previous study demonstrated that a certain amount of IMI accumulated on the surface of pre-killed Gammarus (Huang et al., 2021). In addition, Dalhoff et al. (2020) distinguished the sorbed fraction and the internalised fraction of cypermethrin for several aquatic species, including *G. pulex*, and they found a significant fraction of the total measured body concentration to be adsorbed to the surface of the organisms (Dalhoff et al., 2020).

Generally, FPF is not likely to bioconcentrate or bioaccumulate in aquatic organisms due to its low log K_{OW} value of 0.08 (Carleton, 2014). Our low BCF value results, which were less than 3 L/kg for *C. dipterum* and 50 L/kg for *G. pulex,* were consistent with this statement (Table 2).

The metabolite of interest (6-CNA) was measured but not detected in this study. Based on previous studies, 6-chloronicotinic acid (6-CNA) could be one of the biotransformation metabolites of FPF, and it was observed in soil and rats (Bayer, 2012). However, it was not found in our study. To the best of our knowledge, no biotransformation of FPF has been found in bee studies (Nauen et al., 2015a).

3.1.2. Toxicokinetics of FPF and comparison with that of IMI

The 10 μg/L treatment of the acute toxicity test of each species was subsequently used for the TK experiment. During the 2 days elimination period, no mortality occurred, and no eliminated FPF was detected in the water at the end of the elimination period.

The first-order one-compartment toxicokinetic model was fitted for three species. The order of the uptake rate from high to low was *G. pulex*, *C. dipterum* and *A. aquaticus* (Table 3). *G. pulex* eliminated FPF as fast as *C. dipterum,* while *A. aquatics* eliminated the slowest (Table 3).

Comparing the toxicokinetics of IMI and FPF, the uptake rate of FPF was lower than the value of IMI for all three species, while the elimination rate of FPF was faster in *C. dipterum* and *G. pulex* and slower in *A. aquaticus* than IMI (Huang et al., 2021) (Table 3).

In addition, calculated uptake and elimination rates can be used to calculate BCF values as well. In order to differentiate between BCF values based on measured concentrations and BCF values based on rate constants calibrated by TK modeling, we call the latter one bioconcentration factor kinetic (BCF_k), which is calculated directly by dividing the uptake rate by the elimination rate (Huang et al., 2021; Li et al., 2021). Comparing the BCF values based on body residue and water concentrations measured at the end of the chronic test (28 days) in the 10 ug/L treatment (Table 2) with the BCF_k (Table 3), the two values were very similar. In other words, our results indicate that at 10 ug/L, the accumulation of FPF has reached a steady state after 28 days because the BCF was close to *BCFk*.

To our knowledge, no other toxicokinetic studies have been performed with FPF and aquatic arthropods. However, previous pharmacokinetic studies of FPF (Haas et al., 2021) and IMI (Zaworra et al., 2019) with bees revealed that, compared to IMI, FPF uptake was slower through the honey bee cuticle over 24 h and eliminated faster. Their studies were similar to our findings for *C. dipterum* and *G. pulex,* but not for *A. aquaticus*. The only difference between *A. aquaticus* and the other two species was the elimination rate of FPF, which was slower than IMI. This indicated that *A. aquaticus* might bind FPF stronger than IMI.

Moreover, FPF and IMI are chemically different; FPF contains the butenolide group, while IMI contains the N-Nitro-guanidine group as the nAChR agonist (Jeschke et al., 2015a). The kinetic differences between IMI and FPF could be caused by differences in their receptor binding affinity. A previous bee study found that the binding affinity of FPF to honey bee nAChR preparations was 6-times lower than IMI (Haas et al., 2021). In addition, Jeschke et al. (2015) found that FPF reversibly binds and activates endogenous insect nAChRs (Jeschke et al., 2015a). In contrast, the receptor binding of IMI, especially its toxic biotransformation product IMI-olefin, has been reported to appear irreversible in invertebrates (Tennekes, 2011; Huang et al., 2021).

Table 3
Parameters of the first-order one-compartment toxicokinetic model for FPF and IMI in the three tested species.

Species	Compound	Parameter	Value	95 % CI	*Elimination time* t_{95}^{a} *(d)*	BCF_k (L/kg)	R^2	References
		k_u ($L \cdot kg_{ww}^{-1} \cdot d^{-1}$) k_e (d^{-1})						
C. dipterum	FPF	k_u	0.59	0.44–0.77	10.70	2.12	0.98	Present study
		k_e	0.28	0.17–0.39				
	IMI	k_u	2.96	2.62–3.33	74.89	70.11	0.92	(Huang et al., 2021)
		k_e	0.040	$3.4 \cdot 10^{-4}$ - 0.11				
G. pulex	FPF	k_u	1.62	1.47–1.79	14.27	7.76	0.99	Present study
		k_e	0.21	0.17–0.25				
	IMI	k_u	5.21	4.87–5.54	24.96	44.41	0.98	(Huang et al., 2021)
		k_e	0.12	0.11–0.16				
A. aquaticus	FPF	k_u	0.39	0.32–0.51	59.91	7.65	0.99	Present study
		k_e	0.05	0.01–0.14				
	IMI	k_u	1.10	0.87–1.50	6.66	2.52	0.99	(Huang et al., 2022b)
		k_e	0.45	0.33–0.57				

a: $t_{95} = -\ln(1 - 0.95)/ke$.

3.2. Acute toxicity of FPF

3.2.1. The lethal and sublethal acute effect of FPF

The species sensitivities based on 96 h EC_{50} values were ranked from high to low as follows, *C. dipterum* (42.4 μg/L), *G. pulex* (94.2 μg/L) and *A. aquaticus* (137 μg/L) (Table S7). Based on these values, *C. dipterum* was 2 times more sensitive than *G. pulex* and 3 times more sensitive than *A. aquaticus*. However, *C. dipterum* (117 μg/L) was equally sensitive to *G. pulex* (112 μg/L) when based on 96 h LC_{50} values. For *A. aquaticus*, the LC_{50} was higher than our highest treatment concentration (1000 μg/L), making it at least 10 times less sensitive than the other species (Table S7).

Among the three species evaluated in this study, the acute sensitivity of the three species to FPF was ranked similarly to IMI, i.e., more toxic to mayflies than to crustaceans (Roessink et al., 2013). However, the difference between the acute LC_{50} and EC_{50} values of FPF was different from those observed for IMI (Huang et al., 2021). In Huang et al. (2021), the difference between EC_{50} and LC_{50} values of IMI for *C. dipterum* increased over time, resulting in a 4-time difference on day 4. In their study with *G. pulex*, the difference between LC_{50} and EC_{50} values decreased over time but still exhibited a 7-time difference on day 4 (Huang et al., 2021). For FPF, these differences between LC_{50} and EC_{50} were much smaller after 4 days (Table S7). For *C. dipterum*, the LC_{50} value was always 2–3 times higher than the EC_{50} value within the 4 days experimental period, while for *G. pulex*, the LC_{50} value was 2–3 times higher than the EC_{50} value in the first 3 days and on day 4 a difference of a factor of 1.2 was observed (Table S7). The closeness of LC and EC values may be related to the rapid elimination of FPF, or the lack of active metabolites, resulting in no delay in the effect of FPF. See Section 3.3.1 for more discussion.

3.2.2. Acute SSD

Among the selected species (Table 1), the toxic effect of FPF and IMI were similar among crustaceans (*G. pulex*, *A. aquaticus* and *H. azteca*). Compared to IMI, FPF was less toxic to insects (Table 1). We made the species sensitivity distribution (SSD) based on the acute toxicity of 6 species for FPF and 39 species for IMI (Fig. 1). The HC_{05} of FPF was 0.052 (0.025–0.14) μmol/L, while the value of IMI was 0.0014 (3.27e-04–0.06) μmol/L, corresponding to 15 and 0.36 μg/L, respectively.

FPF was 37 times less acutely toxic to aquatic arthropods than IMI (Fig. 1). To our knowledge, the highest measured environmental concentration of FPF is 0.16 μg/L which was recorded in a watershed of the

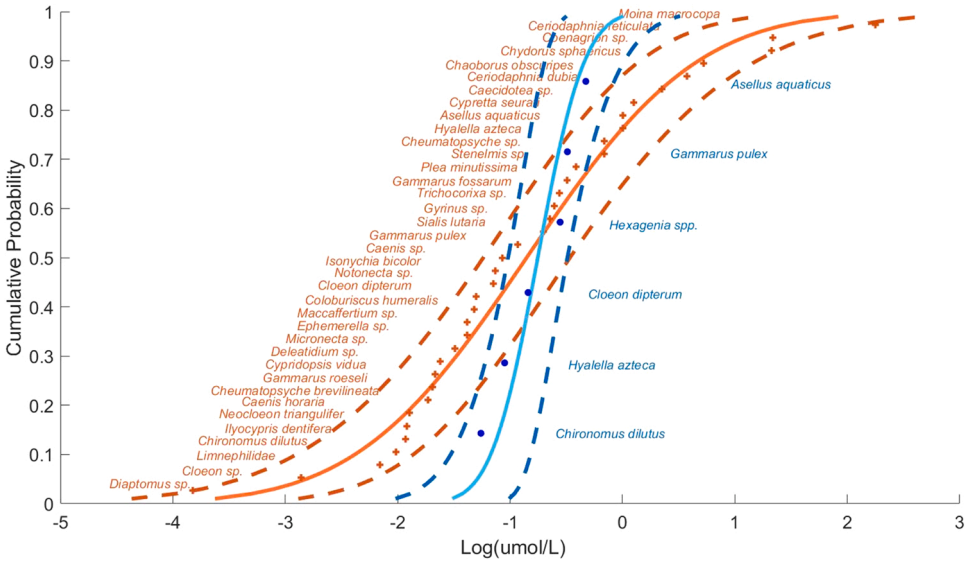

Fig. 1. Species sensitivity distributions for acute toxicity based on the results of laboratory single species tests performed with 39 different aquatic invertebrate species for imidacloprid (red) and 6 species for flupyradifurone (blue). The dots are the experimental data, the solid lines are the fitted curve, and the dash lines denote the corresponding 95 % confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Great Lakes basin, and the geometric mean in 6 watersheds near cropland was 0.018 μg/L (Metcalfe et al., 2019). It was also detected in streams, with IMI being detected in 33 % of the samples, while FPF was detected in 13 % of samples, with the highest concentration of 0.11 μg/L (Sanford and Prosser, 2020). Based on our SSD result and current environmental monitoring results, we could conclude that FPF is safe to date for acute exposure to aquatic arthropods. However, more toxicity assessments of FPF should be performed to incorporate more aquatic arthropods into the SSD, as it currently only consists of 6 data points, which is too limited as a minimum number of 13 taxa is recommended (Carr and Belanger, 2019). In addition, as more research encourages the inclusion of FPF in environmental monitoring programmes, we will have a better understanding of its environmental concentrations in the future (Kandie et al., 2020; Sanford and Prosser, 2020).

3.3. Chronic toxicity of FPF

3.3.1. The lethal and sublethal chronic effect of FPF on C. dipterum and G. pulex

During the 28 days experiment, some emergence of *C. dipterum* was observed. The first emergence occurred on day 14 in the control group, the second emergence occurred on day 17 in the control group, and the rest emergence happened in the last week (day 21–28). The percentage of the emerged individuals was not different among treatments (Fig. S11). A further description and discussion on the emergence are provided in the supporting information Text S5.

The effective concentrations for mortality and immobility calculated for the different sampling dates of FPF for *C. dipterum* were close, and the value remained stable over time (Table S8). This study found that the 28d EC_{10} and EC_{50} values were 7.5 μg/L and 9.1 μg/L, respectively (Table S8). In a previous study, the 28d EC_{10} and EC_{50} of IMI for *C. dipterum* were 0.03 μg/L and 0.1 μg/L, respectively, with the toxicity decreasing from a 96 h EC_{50} of 1.0 μg/L to a 28d EC_{50} of 0.1 μg/L (Roessink et al., 2013). Hence, compared to IMI, the chronic toxicity of FPF was much lower than that of IMI and did not increase over time as observed for IMI.

For *G. pulex*, control mortality was 23 % at the end of the experiment and 12 % on day 21. A discussion of the control mortality is provided in the supporting information (Text S6). The effective concentrations of FPF for mortality and immobility of *G. pulex* were close, and these values decreased over time (Table S8). The 28d EC_{10} and EC_{50} values were 2.9 μg/L and 10.6 μg/L, respectively (Table S8). The chronic toxicity of FPF to *G. pulex* was similar to that of IMI as the 28d EC_{10} and EC_{50} of IMI to *G. pulex* were 3.0 (1.2–7.6) μg/L and 15.4 (9.80–24.1) μg/L, respectively (Roessink et al., 2013), while the molar mass of FPF is only 13 % higher than that of IMI.

The large discrepancy between EC and LC values is a remarkable feature of the toxicity of IMI (Roessink et al., 2013). IMI resulted in immobilisation, but the organisms remain alive for quite some time, also known as delayed effects due to cumulative toxicity (Maloney et al., 2017; Li et al., 2021). This delay in mortality of IMI was attributed to the production of a bioactive metabolite over time (Huang et al., 2021). In the present study, this difference between EC and LC values was not significant for FPF, at least for the two tested species, *G. pulex* and *C. dipterum*. This might be explained by the lack of production of the active metabolite, as we detected none in the internal concentration measurements (Section 3.1).

3.3.2. The food consumption inhibition of FPF on G. pulex

We observed the inhibition of FPF on the food consumption for *G. pulex*, especially after two weeks (Fig. 2). During the first two weeks, none of the treatments significantly inhibited the food consumption compared to the control group (Fig. 2A). During the latter two weeks, the 1 and 3 μg/L treatments significantly inhibited ($P < 0.05$) the food consumption compared with the control group (Fig. 2B). This 28d NOEC of 0.3 μg/L based on food consumption inhibition is a factor of 10 lower than the 28d EC_{10} based on immobilisation (2.9 μg/L) (Table S8; Fig. 2).

Again, since data on the effects of FPF on the food consumption of aquatic species was not available, we compared our results with a honey bee study (Tosi et al., 2021). In their study, Tosi and co-workers provided the first evidence that FPF reduces the food consumption of bees after chronic exposures of 20 days. Furthermore, they found that the inhibition of food consumption caused by FPF became more pronounced over time, even at lower doses (Tosi et al., 2021). We had the same observation in our study that food consumption inhibition only became significant after 2–4 weeks of exposure (Fig. 2). This can be explained by the low uptake rate and fast elimination of FPF (data shown in Section 3.1.2), so it takes longer to exert the effects at low concentrations.

We found two studies (Nyman et al., 2013; Agatz et al., 2014) that studied the effects of IMI on the food consumption of *G. pulex*. They found that the feeding rate of *G. pulex* was significantly reduced after continuous exposure to 30 μg/L IMI for 4 days (Agatz et al., 2014) and 15 μg/L IMI for 14 days (Nyman et al., 2013). However, in their study, the feeding rate was calculated by either the number of disappeared

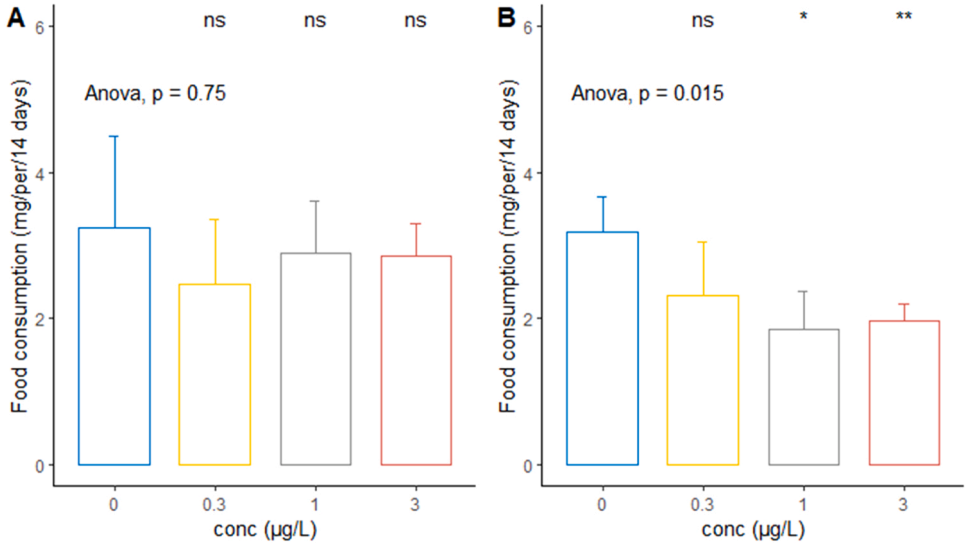

Fig. 2. Food consumption per G. pulex individual for the first two weeks (A) and the latter two weeks (B) in the different treatments (mean ± sd, n = 5 in control, and n = 3 in the treatments).

leaves (Nyman et al., 2013) or the fresh weight of *G. pulex* (Agatz et al., 2014), whereas our study used the decrease in dry weight of leaves per individual as a measure. These differences in approaches make the results difficult to compare in order to assess whether the food consumption inhibition effect of FPF is higher than that of IMI.

Nevertheless, our findings suggest that an inhibitory effect of FPF on *G. pulex* food consumption (NOEC was 0.3 μg/L) may occur at the same order of magnitude as the currently measured maximum environmental concentration of FPF (0.16 μg/L) (Metcalfe et al., 2019). Our findings indicate that FPF could disturb the ecosystem process of leaf litter breakdown via changes in the shredding activity of *G. pulex*.

3.4. The calibration and the prediction of the TKTD for FPF

After calibration on acute data, we used the resulting GUTS-FULL and GUTS-RED models to predict the observed chronic survival for each species. The SD model fitted the acute data better than the IT model both for GUTS-FULL and GUTS-RED, indicated by lower AIC and log-likelihood values (Table S6).

Model predictions of both the GUTS-FULL and GUTS-RED models matched the observed chronic effects not so well for *C. dipterum*, despite NRMSE values being relatively low at 20 % for the GUTS-FULL-SD and 19 % for the GUTS-RED-SD. On visual examination, predictions of the full and the reduced model underestimated the onset of chronic toxicity to a certain extent (Figs. 3B, S5B). For *G. pulex*, the chronic toxicity predictions matched better with the observations with NRMSE values of 17 % for GUTS-FULL-SD and 15 % for GUTS-RED-SD (Figs. 4B,S6B). Surprisingly, the GUTS-RED predicted the chronic toxicity better than the GUTS-FULL model for *G. pulex*, predominantly visible in the better fit for the 10 μg/L treatment (Fig. S6, B5). The fact that the GUTS-RED predicted the chronic toxicity better than the GUTS-FULL model for *G. pulex* is surprising at first look; however, it could give an indication that measured internal concentrations were not an ideal predictor of the concentrations at target sites. Here, the additional information included in measured and modelled internal concentrations did not increase the predictive capacity, which is also seen in the lower NRMSE values for the reduced models (Table S6).

In general, GUTS modeling could predict the mortality of *G. pulex* under chronic exposure reasonably well based on acute effect data, while for *C. dipterum* the prediction was possible only with limited accuracy. The IT models underestimated the observed effects under chronic exposure for both tested species (Figs. S7–S10).

To the best of our knowledge, only one other study has explored the calibration and validation of the GUTS model for the toxicity of FPF to aquatic invertebrates (Gergs et al., 2021). They performed the calibration and validation of the GUTS-RED for three compounds, imidacloprid, thiacloprid, and flupyradifurone with *Chironomus riparius*, and they found that the validation of FPF was better than that of the other two compounds. This finding indicates that the chronic effect of FPF on *C. riparius* could be predicted based on the mechanisms exerted in the acute study (Gergs et al., 2021). Our calibration and prediction result of *G. pulex* was in line with their study.

In addition, a similar calibration and prediction study has been performed for IMI with *C. dipterum* and *G. pulex* (Huang et al., 2021). In their study, a GUTS-FULL model was calibrated by using 4 days acute toxicity test data plus a toxicokinetic test, and they compared the experimental 28 days EC_{50} values (Roessink et al., 2013) with predictions based on the GUTS-FULL model. They found that the predicted chronic toxicity value was higher than the experimental results, which could be explained by the fact that the slower eliminated active biotransformation metabolite, IMI-ole, was not included in the model prediction. Our results of FPF indicate that before performing GUTS modeling to extrapolate acute toxicity data to chronic toxicity, it should be investigated whether a bioactive metabolite is present.

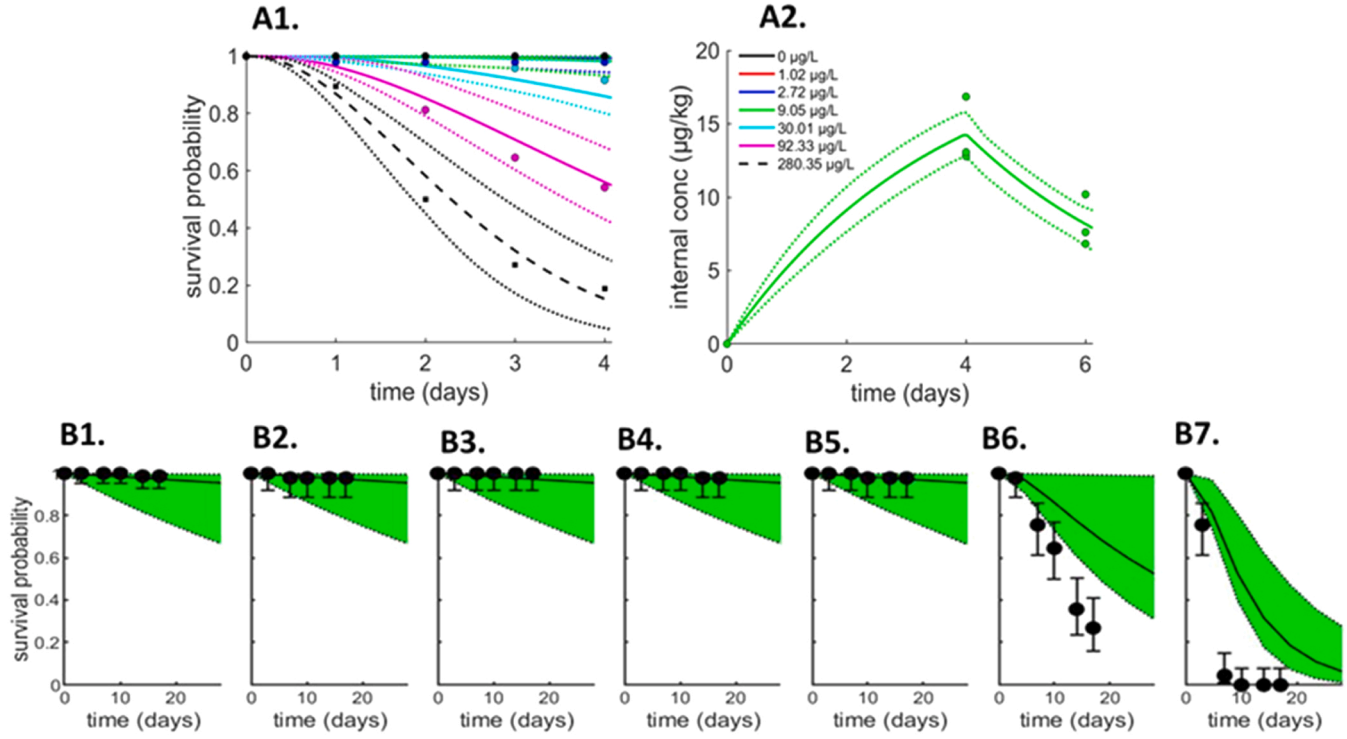

Fig. 3. The calibration (A) and prediction (B) of GUTS-FULL-SD for flupyradifurone (FPF) toxicity to C. dipterum. From left to right, panel A shows the survival fraction in the acute study (A1) and the toxicokinetics of FPF (A2). The dots, dashed lines, and dotted lines represent measured values, fitted values and confidence intervals, respectively. From left to right, panels B show the prediction of chronic survival data in the 0, 0.1, 0.3, 1, 3, 10 and 30 μg/L treatments, respectively. The dots represent the survival data from chronic test; bars represent Wilson score confidence intervals. The line and the green area represent the prediction and the 95 % confidence interval from the GUTS-FULL prediction, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

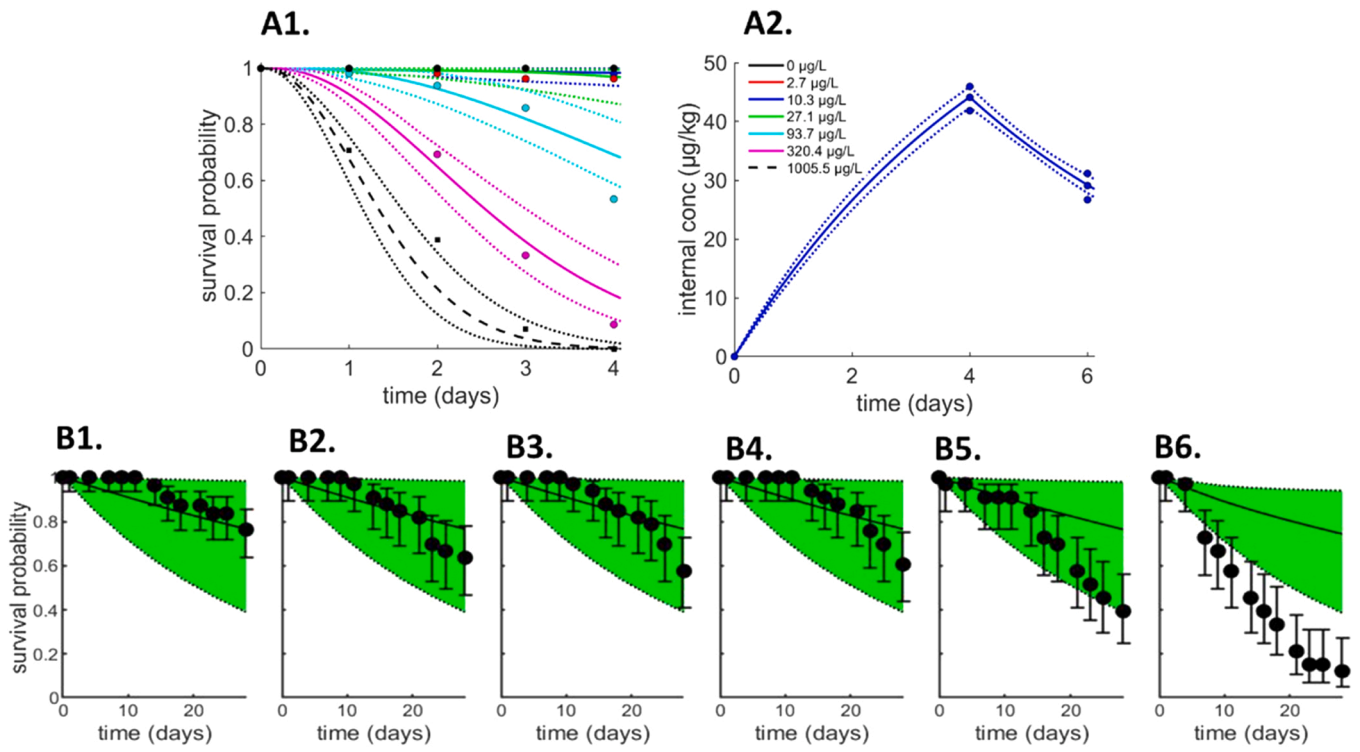

Fig. 4. The calibration (A) and prediction (B) of the GUTS-FULL-SD model of FPF toxicity to G. pulex. From left to right, panel A shows the survival fraction in the acute study (A1) and the toxicokinetics of FPF (A2). The dots, dashed lines, and dotted lines represent measured values, fitted values and confidence intervals, respectively. From left to right, panels B show the prediction of chronic survival data in the 0, 0.3, 1, 3, 10 and 30 µg/L treatments, respectively. The dots represent the survival data from chronic tests; bars represent Wilson score confidence intervals. The line and the green area represent the prediction and the confidence interval from the full-GUTS prediction, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It should be noted again that the emergence of *C. dipterum* was not included in the GUTS model. A more detailed model, such as a dynamic energy budget model (DEB), which includes the life cycle and energy allocation of organisms, could be used to further investigate FPF effects on sublethal endpoints like growth, reproduction, and emergence (Sherborne and Galic, 2020).

4. Conclusions

We assessed the acute and chronic toxicity of FPF to aquatic arthropod species and compared it with the toxicity of IMI. We found that, compared to IMI, *C. dipterum* and *G. pulex* show a slower uptake and faster elimination rates for FPF. FPF was less acutely toxic than IMI based on the HC_{05} values. The chronic 28d EC_{50} and EC_{10} values of FPF were higher or similar to those of IMI. However, FPF inhibited the food consumption of *G. pulex* at a concentration of the same order of magnitude as the current environmental realistic concentration (NOEC = 0.3 µg/L). More environmental monitoring studies of FPF should be performed to know the environmental concentration of FPF better. Overall, chronic effects of FPF on aquatic arthropod species seem to be predicted better than those of IMI, at least for the tested species.

CRediT authorship contribution statement

Anna Huang: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Annika Mangold-Döring:** Conceptualization, Methodology, Writing- Reviewing. **Andreas Focks:** Conceptualization, Modelling, Writing- Reviewing, Supervision. **Chong Zhang:** Methodology. **Paul van den Brink:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the raw data of this study, see https://data.mendeley.com/datasets/52bfvs5vyn.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113977.

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