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The effect of temperature on toxicokinetics and the chronic toxicity of insecticides towards *Gammarus pulex*

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HIGHLIGHTS

- Mortality due to imidacloprid (IMI) and flupyradifurone (FPF) exposure increases with increasing temperature.
- Temperature influences the toxicokinetics of IMI and FPF.
- Elevated temperatures exacerbated the adverse effects of chemicals on sublethal endpoints.
- Temperature should be integrated within the ecological risk assessment of insecticides.

GRAPHICAL ABSTRACT

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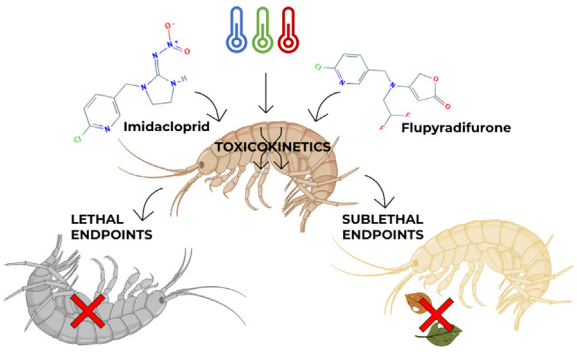

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ABSTRACT

A comprehensive understanding of chemical toxicity and temperature interaction is essential to improve ecological risk assessment under climate change. However, there is only limited knowledge about the effect of temperature on the toxicity of chemicals. To fill this knowledge gap and to improve our mechanistic understanding of the influence of temperature, the current study explored toxicokinetics and the chronic toxicity effects of two insecticides, imidacloprid (IMI) and flupyradifurone (FPF), on *Gammarus pulex* at different temperatures (7–24 °C). In the toxicokinetics tests, organisms were exposed to IMI or FPF for 2 days and then transferred to clean water for 3 days of elimination at 7, 18, or 24 °C. In the chronic tests, organisms were exposed to the individual insecticides for 28 days at 7, 11, or 15 °C. Our research found that temperature impacted the toxicokinetics and the chronic toxicity of both IMI and FPF, while the extent of such impact differed for each insecticide. For IMI, the uptake rate and biotransformation rate increased with temperature, and mortality and food consumption inhibition was enhanced by temperature. While for FPF, the elimination rate increased with temperature at a higher rate than the increasing uptake rate, resulting in a smaller pronounced effect of temperature on mortality compared to IMI. In addition, the adverse effects of the insecticides on sublethal endpoints (food consumption and dry weight) were exacerbated by elevated temperatures. Our results highlight the importance of including temperature in the ecological risk assessment of insecticides in light of global climate change.

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1. Introduction

There is growing awareness that climate change and other anthropogenic activities, such as removal of riparian vegetation, may cause surface water warming and affect the toxicity of many chemicals to aquatic organisms (Noyes and Lema, 2015; Polazzo et al., 2022). Thus, the influence of temperature needs to be incorporated into the ecological risk assessment (ERA) of chemicals (Noyes and Lema, 2015; Van den Brink et al., 2018). In protocolised toxicity studies, researchers are required to use a set temperature in both acute and chronic tests (e.g., between 18 and 22 °C for Daphnia (OECD, 2004, 2012)), which can underestimate or overestimate the effects of chemicals for realistic temperature scenarios. Therefore, studying a range of temperatures, including low and high temperatures, is critical to understanding the interactive effect of temperature on chemicals (Hooper et al., 2013; Moe et al., 2013).

The key to understanding chemical toxicity is comprehending its toxicokinetics inside the organism. Toxicokinetic (TK) processes are the chemical uptake into the organism, its biotransformation inside the organism, and finally, the elimination of the chemical out of the organism. The temperature can influence these kinetic processes by speeding up the underlying physiological mechanisms. The frequently proposed mechanism for the uptake rate is an increase in the organism's metabolic activity with rising temperatures, leading to an increase in oxygen demand (Pörtner, 2010). Additionally, the oxygen concentration in water decreases at higher temperatures. To counter the oxygen deficiency, the organism may increase its ventilation rate (Camp and Buchwalter, 2016) simultaneously increasing the uptake of contaminants present in the water (Buchwalter et al., 2003). The temperature influence on the elimination rate can be the same mechanism as the uptake (i.e., when the compound is eliminated via diffusion across the gills), or it can be driven by the temperature influence on biotransformation. The biotransformation of neonicotinoids in invertebrates is mediated by microsomal cytochrome P450 monooxygenases (CYP450s) (Honda et al., 2006; Casida, 2011). Like all enzymes, these enzymes have a temperature at which their catalytic performance is optimal (T_{opt}). Thus, if temperature increases from below their T_{opt} towards it, we can expect increased biotransformation rates. However, only limited knowledge exists on the influence of temperature on TK processes of imidacloprid (IMI) (Camp and Buchwalter, 2016), while this has not been studied to our knowledge for flupyradifurone (FPF), which is one of the alternative to IMI (Huang et al., 2022a).

In addition, current ecotoxicity studies are mainly conducted over a short period (4 to 7 days) and focus on lethal effects (Schuijt et al., 2021). For example, it was previously shown that the acute lethal effects of IMI on several aquatic arthropods were higher at higher temperatures (Camp and Buchwalter, 2016). However, the life-history traits of organisms, such as feeding rate, growth, and reproduction, may be influenced by temperature as well and also govern the population dynamics (Vellinger et al., 2012; Macaulay et al., 2020; Tran et al., 2020). Hence, the impact of temperature on the long-term effects of chemicals on lethal and non-lethal endpoints needs further investigation.

The present study uses IMI and FPF because of their high water solubility (log K_{ow} value 0.57 for IMI and 1.2 for FPF) and environmental persistence. The dissipation of IMI in water merely depends on photolysis with a half-life ranging from 1 day to 150 days (Morrissey et al., 2015; Sumon et al., 2018). For FPF, the half-life in an outdoor microcosms study was 63.9 days (Carleton, 2014; Sanford and Prosser, 2020). IMI is one of the most used insecticides worldwide (Macaulay et al., 2020). The environmental concentration of IMI in surface water ranges from ng·L^{-1} to μg·L^{-1} levels (Morrissey et al., 2015; Sánchez-Bayo et al., 2016). Many studies showed the toxicity of IMI towards aquatic systems (Morrissey et al., 2015). Based on an acute species sensitivity distribution (SSD), the hazardous concentration 5th percentiles (HC_{05}) of IMI was reported as 0.36 μg·L^{-1} in a recent study (Huang et al., 2022a). Besides, IMI is well known for its delayed or cumulative adverse effect caused by generating a bioactive metabolite, imidacloprid olefin (IMI-ole) (Huang et al., 2021). The new butenolide pesticide FPF is considered a safer alternative to IMI (Giorio et al., 2017). To the best of our knowledge, there is only little monitoring data of FPF with the highest detected concentration of 0.16 μg·L^{-1} in a watershed of Canada (Metcalfe et al., 2019). Lately, several studies have found that FPF showed acute toxic effects on aquatic species (Bartlett et al., 2018, 2019; Maloney et al., 2020; Huang et al., 2022a). The HC_{05} of FPF was 15 μg·L^{-1} based on the acute SSD (Huang et al., 2022a). Current knowledge of the biotransformation FPF suggests that it has no or less toxic metabolites (Jeschke et al., 2015).

Gammarus pulex belongs to the family of amphipod crustaceans that spends its entire life cycle in water and is one of the most common and essential invertebrate species in streams of Northern Europe (Cold and Forbes, 2004). As a detritivore, it plays a vital role in the degradation of leaf litter in aquatic systems (Olivier Dangles et al., 2004). A previous study has found that IMI could inhibit the feeding rate of *G. pulex* (Agatz et al., 2014). Moreover, *G. pulex* is a frequently used aquatic species in toxicity studies since it is sensitive to temperature and chemicals (Sutcliffe et al., 1981; Vellinger et al., 2012; Agatz and Brown, 2014).

The objective of this study was to explore the influence of temperature on the effect of the two insecticides on *G. pulex*. As temperature influences on the TK may change the actual internal exposure to the chemical and, therefore, indirectly affect the apparent chemical's toxicity to the organism, we conducted both a systematic evaluation of the temperature influences on TK and toxicity. The choice of temperature ranges was based on the thermal windows of *G. pulex* (Maazouzi et al., 2011; Moenickes et al., 2011) and the yearly field temperature of the site where we collected the organisms (ranging from 7 to 24 °C, depending on exposure time). We hypothesised that increasing temperature would enhance the effect of insecticides on both lethal and sublethal endpoints (immobility, food consumption, growth, fresh weight, dry weight, and water content).

2. Materials and methods

2.1. Chemicals and test organisms

Imidacloprid (IMI; CAS: 138261-41-3), its bioactive metabolite imidacloprid-olefin (IMI-ole; CAS: 115086-54-9), flupyradifurone (FPF; CAS: 951659-40-8) and its metabolite 6-chloronicotinic acid (6-CNA; CAS: 5326-23-8) were obtained from Sigma Aldrich. Stock solutions of IMI and FPF were dissolved into MiliQ water. Imidacloprid-d4 (IMI-d4; CAS: 1015855-75-0) was used as an internal standard during the analytical measurements of any organism samples. Stock solutions of IMI-d4 (200 μg·mL^{-1}) were dissolved into 2 % acetone (v:v) to ensure that the compound was fully dissolved.

Gammarus pulex was collected from an uncontaminated brook, the Heelsumse brook (coordinates 51.973400, 5.748697) in July (for TK experiments) and December (for chronic experiments of IMI and FPF) of 2020. This brook is groundwater-fed and cool in summer. That brook's yearly water temperature range is from 4 °C to 17 °C based on personal observation. The experimental temperatures 7, 11, 15, 18, and 24 °C are within the thermal tolerance of the organism, as *G. pulex* has a temperature optimum of 10–20 °C and an upper thermal limit of 27–33 °C and an lower limit in previous experiments as 0 to 5 °C (Sutcliffe et al., 1981; Maazouzi et al., 2011; Moenickes et al., 2011).

2.2. Toxicokinetic (TK) experiments

Juvenile organisms with an average length of 6.87 mm (sd: 0.96 mm) were brought to the laboratory, counted, and separated into three groups. After 12 h acclimatization to the laboratory conditions at catchment temperature (14 °C) and a 12:12 h light:dark cycle, the individual water bath compartments were adjusted to 7, 18, and 24 °C at a rate of 0.5 °C per hour. The organisms were kept at these experimental temperatures for another minimum of 24 h. During the whole acclimatization procedure, the organisms were fed with *Populus* leaves ad libitum.

During the experiment, a replicate system consisted of ten individuals in a 1.5 L glass jar filled with 1 L groundwater retrieved from the *Sinderhoeve*

experimental station (the Netherlands; www.sinderhoeve.org). We placed a piece of metal mesh in each jar as a substrate for the organisms to increase the surface area on which they can sit on or attach to, aiming to prevent cannibalistic behavior. During the uptake phase, the organisms were exposed to either 17.62 (sd: 0.49) $\mu g \cdot L^{-1}$ IMI or 18.24 (sd: 1.00) $\mu g \cdot L^{-1}$ FPF. The exposure concentrations were 0.1 times the 50 % effective concentration after 2 days of exposure (48 h, EC_{50}) of *G. pulex* for IMI and FPF, respectively (Huang et al., 2021; Huang et al., 2022a).

Internal concentrations were measured after 6, 24, and 48 h in the uptake phase and after 72, 96, and 120 h in the elimination phase. After 48 h, all remaining organisms were transferred to new jars with uncontaminated groundwater for the elimination phase. At each time point, three exposed replicates were sampled destructively. Organisms' survival was monitored throughout the whole experiment. At each time point, the respective jars were removed from the water bath, the organisms were washed with demineralized water, quickly dried on a paper towel, weighed to get the fresh weight, and frozen at −20 °C before further analysis of the internal concentration of the chemicals in the organisms.

2.3. Toxicokinetic (TK) modelling

Considering the generation of metabolites, different TK model types were used for IMI and FPF. As no metabolites were measured in the organisms samples exposed to FPF, a simple one-compartment first-order TK model was calibrated on the measured internal concentrations for each temperature (Supplementary data Text S1, Table S1, Eq. (1)). For the exposures to IMI, no metabolite was detected at 7 °C, so this data set was also calibrated to a simple one-compartment model. During the elimination phase in the experiments conducted at 18 and 24 °C, IMI-ole was detected. Thus, these results were calibrated to a one-compartment TK model with metabolite, considering the biotransformation of IMI into IMI-olefin (Supplementary data Text S1, Table S1, Eqs. S2–3).

All model calibrations were performed in the software Matlab (2020b), starting from scripts available within the Bring Your Own Model (BYOM) modelling platform (www.debtox.info/byom.html, Version 5.2). Matlab scripts that were used can be downloaded from GitHub (https://github.com/NikaGoldring/Toxicokinetic-models-for-pesticides-in-Gammarus-pulex). The model parameters (Supplementary data Text S1, Table S1) were estimated based on the Nelder-Mead simplex optimization algorithm provided in the BYOM platform and their confidence intervals were generated applying the likelihood region method.

2.4. Chronic experiments

Chronic experiments of 28 days were performed with *G. pulex*. After field collection, healthy juvenile individuals (without parasite seen as an orange dot on the back) with similar lengths (around 5 mm) were randomly selected and put into three buckets with a mixture of field water and pre-aerated groundwater from the *Sinderhoeve* experimental station (the Netherlands; www.sinderhoeve.org). *G. pulex* was acclimatized in three water bath sections at field temperature (11 °C) for two days. Afterward, the temperature in each section was gradually increased or decreased to the experimental temperatures at a rate of 0.5 °C per hour. The intended temperatures were 7 ± 1 °C, 11 ± 1 °C and 15 ± 1 °C. After each section reached its experimental temperatures, *G. pulex* was acclimatized for at least two days. During the acclimation period, organisms were fed leached *Populus* leaves ad libitum. A light:dark regime of 12:12 h was used. Previous chronic pilot study (at 7, 18 and 24 °C) found that the control mortality at 18 °C and 24 °C was above 20 % after 28 days experiment (data not shown), thus we selected 7, 11 and 15 °C in the formal chronic experiment.

Experiments were performed with three replicates per treatment level and five replicates for controls. In addition, 5 concentration levels plus control were used. The experimental setups are in line with OECD requirements (OECD, 2004). At the start of the experiment, each replicate consisted of 11 individuals added to 1 L groundwater, after which a volume was dosed to reach a concentration of 0, 0.3, 1, 3, 10 or 30 $\mu g \cdot L^{-1}$ for both IMI and FPF. The selection of the concentration was based on previous chronic IMI and FPF test results at 18 °C (Roessink et al., 2013; Huang et al., 2022a). Gentle aeration was provided in the test systems. Immobility and mortality were monitored every 2 to 3 days during the experiment based on a method described in Roessink et al. (2013) (Roessink et al., 2013). Individuals were scored as immobile when no movement of any kind, except for the heart, was observed for a period of 20 s and were scored as dead when no response of any kind was observed during 3 to 5 s of gentle stimulation using a Pasteur's capillary pipette. Dead organisms were removed from the test vessels. Water samples were taken every week to measure the exposure concentrations of the analytes (Section 2.6), and the stability of chemicals were verified. In addition, we renewed the system every two weeks, all remaining animals were genteelly transferred to new medium.

The food consumption of *G. pulex* was measured every two weeks in this study. The pre-treated (i.e., leached in stream water then progressively acclimatized to the test water before use) *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 (McGrath et al., 2007). Two pieces of dry leaf discs were provided for every replicate. The dry weight of the leaves of each replicate was recorded before adding them into the system. The dry leaves were added to each replicate 3 days before the organisms and the chemical to allow the leaves to soak in the 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. Two jars with only stainless-steel mesh and conditioned *Populus* leaves, and no *G. pulex*, were installed to estimate the microbial degradation of the leaves. The food consumption of each replicate was calculated as the difference between the initial leaf's weight and the remaining leaf's weight after the loss was corrected for microbial degradation (Eqs. (1) and (2)).

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

$$L_e = L_i - L_f - Lc. \tag{1}$$

where $L_i$and L_f are the initial and final dry mass (mg) of leaves, Lc is the average dry mass loss of the control group accounting for microbiological degradation.

To obtain the 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. (2)). The food consumption per organism (F_{total}) after two weeks was calculated as:

$$F_{total} = \frac{L_e}{\frac{\Sigma(n_1+n_2+n_3+n_4+n_5+n_6)}{6}} \tag{2}$$

where n is the number of remaining organisms at each observation time (1–6), observed on days 1, 4, 7, 9, 11, and 14 in the first two weeks and on days 16, 18, 21, 23, 25, and 28 for the last two weeks.

To obtain the food consumption F per individual per day, we divided F_{total} by the two weeks period (Eq. (3)).

$$F = \frac{F_{total}}{14} \tag{3}$$

The internal concentration, fresh weight, dry weight, and water content of the remaining organisms were measured at the end of the experiment (28 day). For fresh weight, individuals from the same jar were dabbed dry and weighed on a microbalance (0.1 mg). Then the samples were frozen for 12 h at −20 °C and freeze-dried for ≥24 h, after which dry weight was measured. The water content of the organisms from the same jar was calculated by the difference between the fresh and dry weights. The internal concentration measurement is described in Section 2.6.

The physicochemical water parameters, dissolved oxygen, pH, electrical conductivity, and temperature were measured only in the control and the

highest treatment weekly. The results are provided in the raw data of water quality parameters in Mendeley data (Huang et al., 2022b).

2.5. Size measurement of Gammarus pulex

The initial size of *Gammarus pulex* was measured in both TK and chronic experiment. 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. S5). 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 (Fig. S5). In the chronic experiment, after 28 days experiment, the size of the remaining organisms was measured too, and the results were presented in Fig. S6. The initial size of *G. pulex* in TK experiment was 6.9 ± 0.9 mm, and in chronic experiment, the initial size in the FPF experiment was 5.7 ± 0.7 mm, while it was 4.2 ± 0.7 mm in the IMI experiment (Huang et al., 2022b).

Note that the timing of collection may affect the results, but we exclude this influence with two reasons. The first one is in both TK and chronic experiment, we selected similar sized organisms to ensure their consistence (age is not applicable as we worked with organisms from field catchment); secondly, TK experiment were conducted in May, and chronic experiment were conducted in December, both animals can be considered a "summer generation" which have not experienced the cold winter in Netherland (which is during Jan-April).

2.6. Chemical analyses

Groundwater and surface water have been analysed by LC/MS-MS to confirm the absence of all the tested analytes. The light in the experiment did not contain ultraviolet light to prevent the photodegradation of FPF and IMI which was confirmed by the analytical measurement with LC/MS-MS. The chemical stability of both IMI and FPF in the 2 days TK and the 28 day chronic experiments was verified by the water samples measurement performed at every timepoint in TK tests or every week in chronic tests (Huang et al., 2022b).

All water and organism samples were analysed by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) based on the measurement of IMI as described by (Huang et al., 2021). 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 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 $mL{\cdot}min^{-1}$. The mass spectrometer was operated using an Agilent jet stream electrospray ionization source 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 was set at 300 °C. The compounds were detected in the multiple reaction monitoring using two transitions per compound. The MS/MS transitions of all compounds are provided in Supplementary data in Text S2, Table S4.

Injected samples were quantified by peak area using the calibration curve constructed from the calibration standards included in the same sample sequence. Agilent Masshunter software (version 8.0) was used for instrument control and data acquisition. The extraction recovery of FPF and IMI in the organisms, evaluated at two concentrations by spiking them into the clean organisms, was acceptable based on recovery and repeatability. The limit of quantification (LOQ) of IMI in water samples was 0.01 $\mu g{\cdot}L^{-1}$, 0.45 $\mu g{\cdot}kg^{-1}$ in organism samples; and the LOQ of FPF in water samples was 0.06 $\mu g{\cdot}L^{-1}$, 4.6 $\mu g{\cdot}kg^{-1}$ in organism samples. The results of all water samples and internal concentration samples were above LOQ. For further information on analysis methods and recovery results, see Supplementary data Text S2, Tables S4–S7.

2.7. Data analysis

2.7.1. Data analysis for mortality and immobility

The mortality and immobility observed during the IMI or FPF experiment period were analysed using generalized linear mixed models (GLMMs) with a binomial error structure (dead vs. alive and immobile vs. alive and mobile) and logistic regression function. Temperature and concentration were fixed factors, the exposure time was the covariate variable, and the jar number was the random factor. In addition, three types of interactions, temperature:concentration, temperature:time, and concentration:time, were included. The interaction of temperature:concentration:time was not significant in any case; thus, it was not included in the GLMM. $\chi 2$ and *p*-values of each factor or interaction in generalized regressions were calculated with the 'car' package (Fox and Weisberg, 2019) in open-source software R version 4.0.5 (Ritz et al., 2015).

In addition, the statistical analysis of NOEC at each temperature was based on the OECD guideline (OECD, 2006). The dose-response relationship for lethal (mortality) and sublethal endpoint (immobility) of IMI and FPF at each temperature was fitted using the log-logistic regression, using GenStat (15th edition, Laws Agricultural Trust; VSN International) (Roessink et al., 2013).

2.7.2. Data analysis for other sublethal endpoints

Sublethal endpoints, such as food consumption and dry weight, were compared among concentration levels and temperatures. The assumption of normally distributed data was evaluated using the Shapiro-Wilk test, while the assumption of equal variance was assessed using a Spearman rank correlation between the residuals and the dependent variable. Data were expressed as mean ± standard deviation. A two-factor analysis of variance (ANOVA) was used to analyse the main effects of temperature and concentration and to detect an interaction between these two variables. The Tukey procedure was used to compare individual means, and a *p*-value of <0.05 was considered statistically significant. Post-hoc pairwise comparisons of significant interactions were made using Tukey contrasts with the TukeyHSD function in the package of "agricolae" in R (version 4.0.5).

3. Results and discussions

3.1. Toxicokinetics of IMI and FPF at different temperatures

All raw data of this study can be obtained from our Mendeley Data (Huang et al., 2022b). The measured internal concentrations of IMI and FPF in *G. pulex* showed a steep increase within the uptake phase (i.e., up to day 2) and decreased during the elimination phase (Fig. 1). The internal concentration of IMI on day 2 was lowest in the 7 °C treatment (with an average concentration across replicates of 62 $\mu g{\cdot}kg^{-1}$, compared to 89 $\mu g{\cdot}kg^{-1}$ at 18 °C) and highest in the 24 °C treatment (with 100 $\mu g{\cdot}kg^{-1}$), suggesting an increased uptake with increasing temperature. The opposite trend was observed for FPF, however, with a smaller effect size, as average concentrations were of the same order of magnitude across temperatures, with 46 $\mu g{\cdot}kg^{-1}$ at 7 °C, 42 $\mu g{\cdot}kg^{-1}$ at 18 °C, and 40 $\mu g{\cdot}kg^{-1}$ at 24 °C. Additionally, the replicated measurements for the internal concentration of FPF at 7 °C showed a larger variation compared to the values measured at the other temperatures (Fig. 1, B1, black triangles). The final internal concentration on day 5 (end of the elimination phase) did not differ much among temperatures for IMI (57 $\mu g{\cdot}kg^{-1}$ at 7 °C, 64 $\mu g{\cdot}kg^{-1}$ at 18 °C, and 59 $\mu g{\cdot}kg^{-1}$ at 24 °C). In contrast, it was lowest in the 24 °C treatment for FPF (with 15 $\mu g{\cdot}kg^{-1}$, compared to 30 $\mu g{\cdot}kg^{-1}$ at 7 and 18 °C). From this, we observe that both compounds' elimination increased with increasing temperature.

The model calibration for each dataset separately (Fig. 1) resulted in three parameter sets, one for each temperature (Supplementary data Text S1, Tables S2–3). These parameters reflect the same pattern previously described by increasing uptake and elimination rate constants with increasing

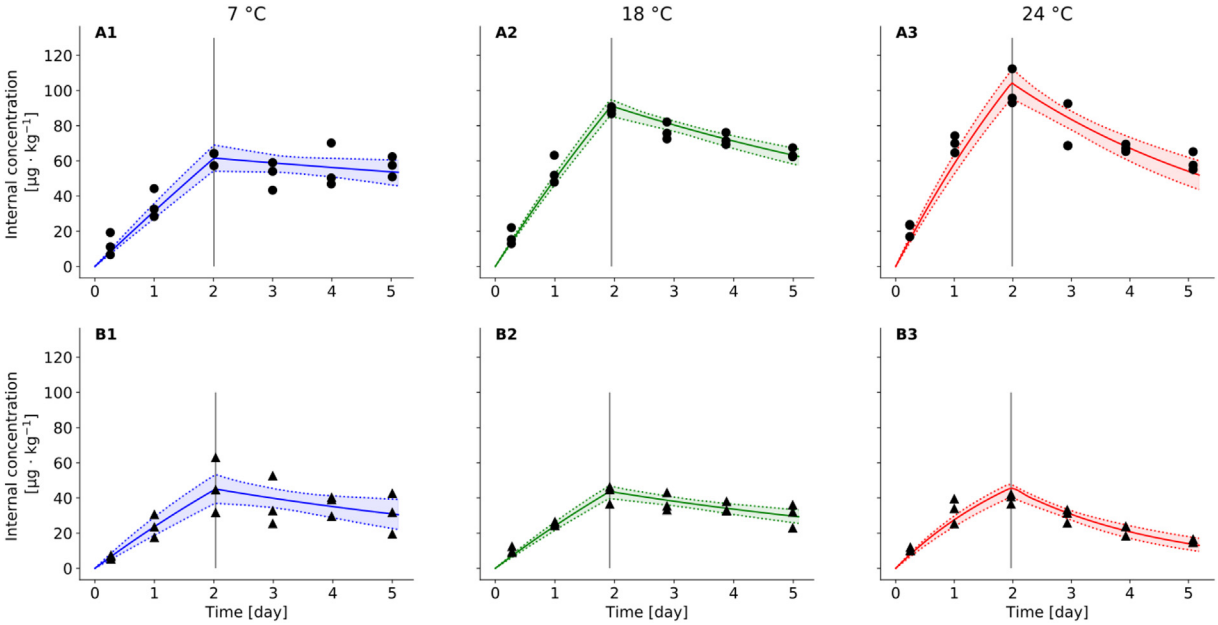

Fig. 1. Internal concentrations and toxicokinetic models of imidacloprid (IMI) and flupyradifurone (FPF) at different temperatures in *Gammarus pulex*. Black dots for IMI (A1–A3) and triangles for FPF (B1–B3) are measured internal concentrations in µg·kg^{-1} at 7, 18, and 24 °C, respectively. Solid lines are the TK model fits with lower and upper confidence intervals (dotted lines). The grey vertical line represents the transition timepoint from the uptake to the elimination phase for each temperature and chemical.

temperature for IMI and FPF. However, the temperature influence was different between parameters (i.e., for IMI, a 2 fold-difference for k_u and a 3.6 fold-difference for k_e) and between the two insecticides (with only a 1.3 fold-difference for k_u and a 3.1 fold-difference of k_e for FPF).

For IMI, increased uptake with increasing temperature was previously reported within a range of aquatic invertebrates (Camp and Buchwalter, 2016). However, no elimination kinetics was evaluated in their study, leaving limited grounds for concluding statements about temperature's effect on the bioconcentration of IMI. In our study, the calibrated parameters could be used to calculate the kinetic bioconcentration factor (BCF_{kin}) for each temperature. For both insecticides, the BCF_{kin} decreased with increasing temperature. However, the BCF_{kin} for FPF at the lowest and the medium temperature was practically the same with 11.2 L·kg^{-1} at 7 °C and 11.0 L·kg^{-1} at 18 °C, while there was a linear decrease observed for IMI (Supplementary data Text S1, Fig. S1).

Another difference between the two insecticides concerns the presence of biotransformation products. No metabolite was detected in the organisms exposed to FPF at any temperature, whereas the metabolite IMI-olefin was detected during the elimination period of the exposures to IMI at 18 and 24 °C (Fig. 2). The metabolite formation rate k_m increased from 0.023 L·kg^{-1}·d^{-1} at 18 °C to 0.051 L·kg^{-1}·d^{-1} at 24 °C. The metabolite elimination rate also increased with increasing temperature from 0.69 L·kg^{-1}·d^{-1} at 18 °C to 0.87 L·kg^{-1}·d^{-1} at 24 °C.

Interestingly, no metabolites were found in the organisms after the 28-day exposure of the chronic toxicity study at any concentration or temperature. For this, we see two possible explanations i) the metabolites are further degraded and thus not measured after 28 days, or ii) the metabolites are not produced at the temperatures used in the chronic experiments (i.e., 15 °C). With regards to the first consideration, to our knowledge, no studies have measured the internal concentrations of long-term exposure to IMI in aquatic species. A previous study found that IMI was biotransformed into IMI-ole in *G. pulex* (Huang et al., 2021). However, this study performed an acute (short time, i.e., 6 days) exposure. IMI-ole can be further biotransformed to 6-CNA in *G. pulex* (Huang et al., 2022c). In addition, IMI can be biotransformed to some intermediates which were not checked for in our LC-MS/MS analysis (Suchail et al., 2001; Fusetto et al., 2017). In line with the second consideration, the temperature may influence biotransformation. While IMI-ole was detected in the TK experiments conducted at 18 and 24 °C, the highest temperature in our chronic study was 15 °C. We speculate that 15 °C might inhibit or delay the biotransformation process of IMI. Nevertheless, we did not measure the internal concentration over time during the chronic exposure experiment. Thus, we are limited in interpreting the internal concentration measured at the end of the 28 days experiment.

3.2. Lethal effects of IMI and FPF at different temperatures

3.2.1. The chronic results after 28 days

In all chronic experiments, the water concentration of FPF and IMI was stable during 28 days experiment and within 20 % deviation with nominal concentration. Thus, the nominal concentration was used in reporting the result below.

The control mortality at each temperature was <20 % (Fig. 3). For the mortality caused by IMI, temperature, concentration, time, the interaction between temperature and concentration, and the interaction between

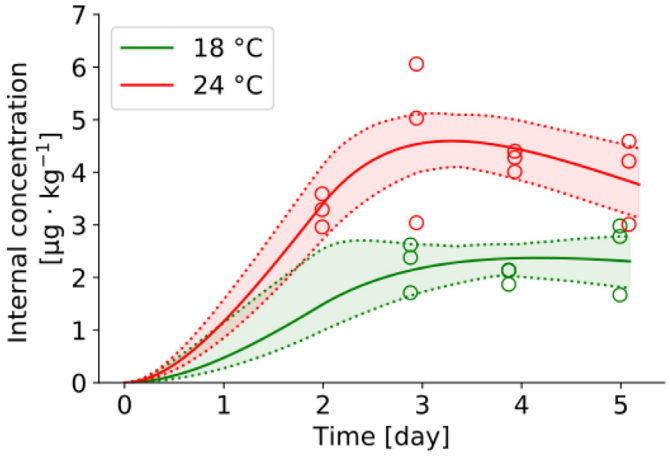

Fig. 2. Internal concentration and toxicokinetic model of imidacloprid-olefin (IMI-ole) at different temperatures in *Gammarus pulex*. Empty green and red circles are measured internal concentrations of IMI-ole in µg·kg^{-1} at 18, and 24 °C, respectively. Solid lines are the TK model fits with lower and upper confidence intervals (dotted lines).

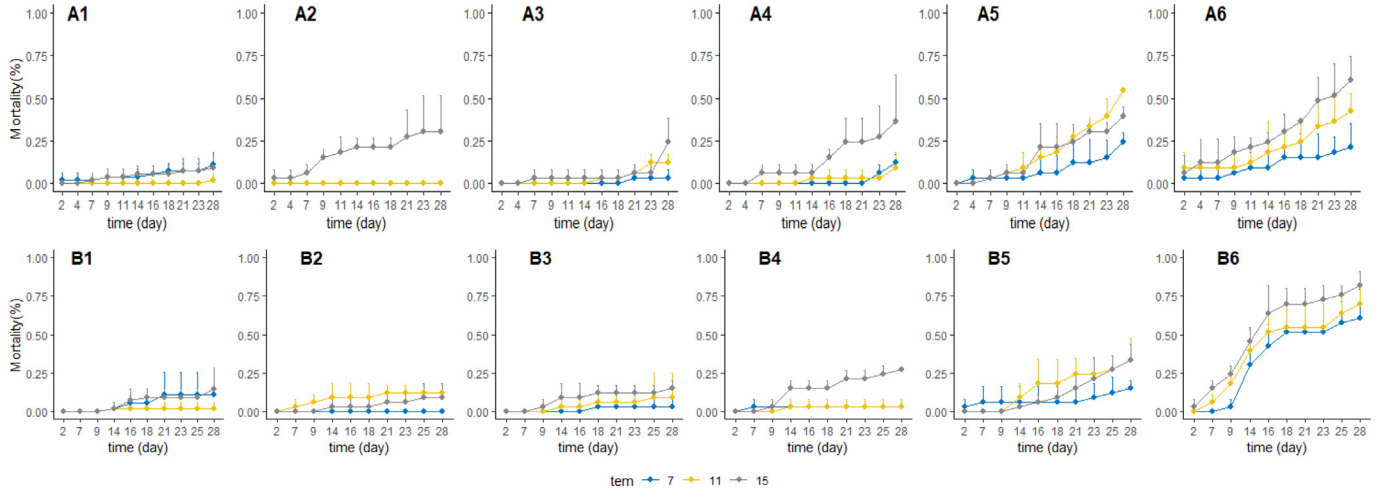

Fig. 3. The mortality under imidacloprid (IMI, A) and flupyradifurone (FPF, B) exposure over time at 7, 11, and 15 °C. Tiles 1 to 6 represent the control group (0 $\mu g{\cdot}L^{-1}$) and the treatments (0.3, 1, 3, 10, 30 $\mu g{\cdot}L^{-1}$), respectively. Plotted are means + s.d. with n = 5 for control groups and n = 3 for treatment groups. tem = temperature in °C.

concentration and time explain a significant part of the variation in the data (Table 1). The NOEC values for mortality and immobility were the same at each temperature; the values were 10 $\mu g{\cdot}L^{-1}$, 0.3 $\mu g{\cdot}L^{-1}$ and <0.3 $\mu g{\cdot}L^{-1}$, for 7 °C, 11 °C and 15 °C, respectively (Table 2). The LC_{10} and LC_{50} values were also presented in Table 2, which decreased with temperature. Regarding FPF, no significant influence of temperature on the lethal effects was detected by the GLMM model (Table 1). However, the LC_{10} and LC_{50} values decreased with temperature (Table 2). Similar to IMI, for FPF, the NOEC values for mortality and immobility were the same at each temperature; the values were 10 $\mu g{\cdot}L^{-1}$, 3 $\mu g{\cdot}L^{-1}$ and 1 $\mu g{\cdot}L^{-1}$, for 7 °C, 11 °C, and 15 °C, respectively (Table 2). Compared to previous studies which conducted chronic tests of IMI (Roessink et al., 2013) and FPF at 18 °C (Huang et al., 2022a), the LC_{50} values at 18 °C were similar to our results at 15 °C in this study, taking into account the relatively large confidence intervals. To be specific, the LC_{50} of IMI was 33.8 (20.9–54.6) μg/L, and the EC_{50} of IMI was 15.4 (9.80–24.1) μg/L at 18 °C (Roessink et al., 2013); the LC_{50} of FPF was 10.6 (2.4–18.8), the EC_{50} of FPF was 10.6 (4.5–16.6) μg/L at 18 °C (Huang et al., 2022a).

Besides, based on the values of NOEC, LC_{10}, and LC_{50} values at each temperature, the observed enhancement of the lethal effects through temperature increase was higher for IMI than for FPF. This finding is consistent with our internal concentration results after 28 days, as we found higher internal concentrations of IMI at higher temperatures (Supplementary data Text S3, Fig. S3). An additional factor for the higher toxicity of IMI could lay in its increased biotransformation into the toxic metabolite IMI-ole. Although no IMI-ole was detected at the end of the chronic experiment, we cannot exclude its presence during the course of the experiment.

Our findings are in line with several studies which conducted acute tests. Previous studies have revealed that increased temperature increases the lethal effects of IMI on several aquatic species. For example, higher temperature decreased the time to immobility for *Isonychia bicolor* (Camp and Buchwalter, 2016), and elevated toxicity of imidacloprid was found under the influence of increasing temperature for *Coloburiscus humeralis* (*Coloburiscidae*) and *Deleatidium* spp. (Macaulay et al., 2020). Insecticides are not the only group of chemicals impacted by temperature. Temperature has been identified as an ion transport modifier, leading to increased influx of mayor ions (e.g., ^{22}NA, $^{35}SO_4$ and ^{45}Ca) with increased temperatures (Orr and Buchwalter, 2020). Additionally, the toxic effects of organic chemicals (Freitas et al., 2019), metals (Sokolova and Lannig, 2008; Bednarska et al., 2017; Haque et al., 2020), and nano plastics (Sulukan et al., 2022) were enhanced by temperature.

Furthermore, a previous study (Sumon et al., 2018) found that a much lower 96 h, EC_{50} value (0.0055 $\mu g{\cdot}L^{-1}$) for an insect species, *Cloeon* sp. at 27.5 °C compared to Roessink et al. (2013) who tested *Cloeon dipterum* at 18 °C and found a 96 h, EC_{50} of 1.02 $\mu g{\cdot}L^{-1}$ (Roessink et al., 2013). Furthermore, the study also indicates that (sub-)tropical aquatic ecosystems can be much more sensitive to imidacloprid compared to temperate ones due to the higher temperature (Sumon et al., 2018). Thus, together with many other studies, the temperature-enhanced toxicity observed in the present study emphasizes the need to consider this abiotic factor in ecological risk assessment (Heye et al., 2019).

3.2.2. The temporal patterns of chronic toxicity

In addition, we also found differences in the temporal toxicity patterns between IMI and FPF. In general, the effects of IMI increased after 14 days of exposure, while the effects of FPF remained almost unchanged (Fig. 3). Specifically, for IMI, we found that the difference between temperatures becomes more significant over time (Fig. 3). For example, at all three temperatures, the effect of 1 $\mu g{\cdot}L^{-1}$ IMI was below 20 % on day 23 and reached nearly 30 % on day 28 at 15 °C, while the other temperatures remained below 20 % (Fig. 3, A3). In the 30 $\mu g{\cdot}L^{-1}$ IMI treatment (Fig. 3, A6), the mortality on day 14 at 7 °C, 11 °C, and 15 °C was 9 %, 18 %, and

Table 1
Generalized linear mixed models (GLMMs) of imidacloprid (IMI) and flupyradifurone (FPF) for mortality and immobility. Effects: tem = temperature, conc = concentration.

Chemical	IMI						FPF					
Effect	Mortality			Immobility			Mortality			Immobility		
	×2	Df	*P*	×2	Df	*P*	×2	Df	*P*	×2	Df	*P*
tem	10.72	2	***4.70E−03***	18.90	2	***7.86E−05***	3.36	2	*0.19*	3.51	2	*0.17*
conc	23.47	5	***2.74E−04***	38.58	5	***2.88E−07***	15.92	5	***7.09E−03***	21.30	5	***7.10E−04***
day	15.32	1	***9.09E−05***	9.82	1	***1.72E−03***	25.37	1	***4.74E−07***	27.81	1	***1.34E−07***
tem: conc	29.28	10	***1.12E−03***	33.50	10	***2.25E−04***	16.96	10	*0.08*	18.46	10	***0.048***
tem: day	0.86	2	*0.65*	15.78	2	***3.75E−04***	1.79	2	*0.41*	1.04	2	*0.60*
conc: day	16.36	5	***5.88E−03***	8.32	5	0.14	11.86	5	***0.04***	8.84	5	*0.12*

Table 2
Toxicity endpoints for imidacloprid (IMI) and flupyradifurone (FPF) at each temperature after 28 days of exposure. Lethal concentrations (LC_{10} and LC_{50}), effect concentrations (EC_{10} and EC_{50}) with their 95 % confidence interval, and the NOEC values obtained for the endpoints mortality, immobility. Temp = temperature in °C and toxicity endpoints in $\mu g \cdot L^{-1}$.

Chemical	Temp	LC_{10}	LC_{50}	EC_{10}	EC_{50}	Mortality	Immobility
IMI	7	9.8 (*)	163.2 (*)	7.8 (1.8–34.4)	93.2 (13.3–650.1)	10	10
	11	1.5 (0.5–4.5)	24.3 (12.0–49.5)	1.5 (0.5–4.3)	20.1 (11.0–36.8)	0.3	0.3
	15	0.1 (0.02–3.0)	30.1 (5.2–174.2)	0.03 (0.0–3.2)	34.5 (4.0–294.9)	<0.3	<0.3
FPF	7	10.3 (4.6–22.9)	33.7 (21.8–52.1)	10.2 (4.7–21.9)	31.4 (21.3–46.3)	10	10
	11	5.5 (2.6–11.7)	18.7 (13.4–26.2)	5.4 (2.6–10.9)	16.9 (12.2–23.3)	3	3
	15	5.1 (2.2–12.1)	16.3 (11.2–23.8)	5.9 (2.8–12.3)	15.9 (11.8–22.5)	1	1

24 %, respectively. However, these values increased to 21 %, 42 %, and 60 % on day 28, respectively.

To the best of our knowledge, only a few studies investigated the influence of temperature on toxicity in a chronic toxicity test (Macaulay et al., 2021). Macaulay et al. (2021) presented clear time-cumulative toxicity of IMI, which first affected mayfly's mobility after 12 days but eventually caused a strong effect (i.e., impairment) at the end of 36 days of exposure, and the heatwave increased the toxicity of IMI.

Our TK results on biotransformation can explain the observed difference in the temporal toxicity pattern between IMI and FPF. For IMI, the biotransformation rate was higher at higher temperatures, resulting in the formation of the toxic metabolite, IMI-ole. Thus, two compounds, the toxic parent compound IMI and its metabolite IMI-ole, which is less toxic but has a slower elimination rate (Huang et al., 2021), affected the organisms. This combined exposure resulted in an accumulating effect over time, also accelerating with increasing temperature. Since, for FPF, no toxic metabolites were detected, there was no such effect (Huang et al., 2022a). Our results indicated that it is essential to consider biotransformation in temperature assessment for toxicity testing, as also indicated in a recent study (Cerveny et al., 2021). In their study, they discovered that the water temperature affects the biotransformation and accumulation of a psychoactive pharmaceutical, temazepam, and its metabolite in aquatic organisms. They further found that the influence of temperature on accumulation and biotransformation was different in different species (Cerveny et al., 2021).

In conclusion, we observed that temperature enhanced the lethal effects of IMI and FPF, where the enhancement extent was greater for IMI than for FPF, and the influence of temperature on IMI became more significant with time due to its biotransformation.

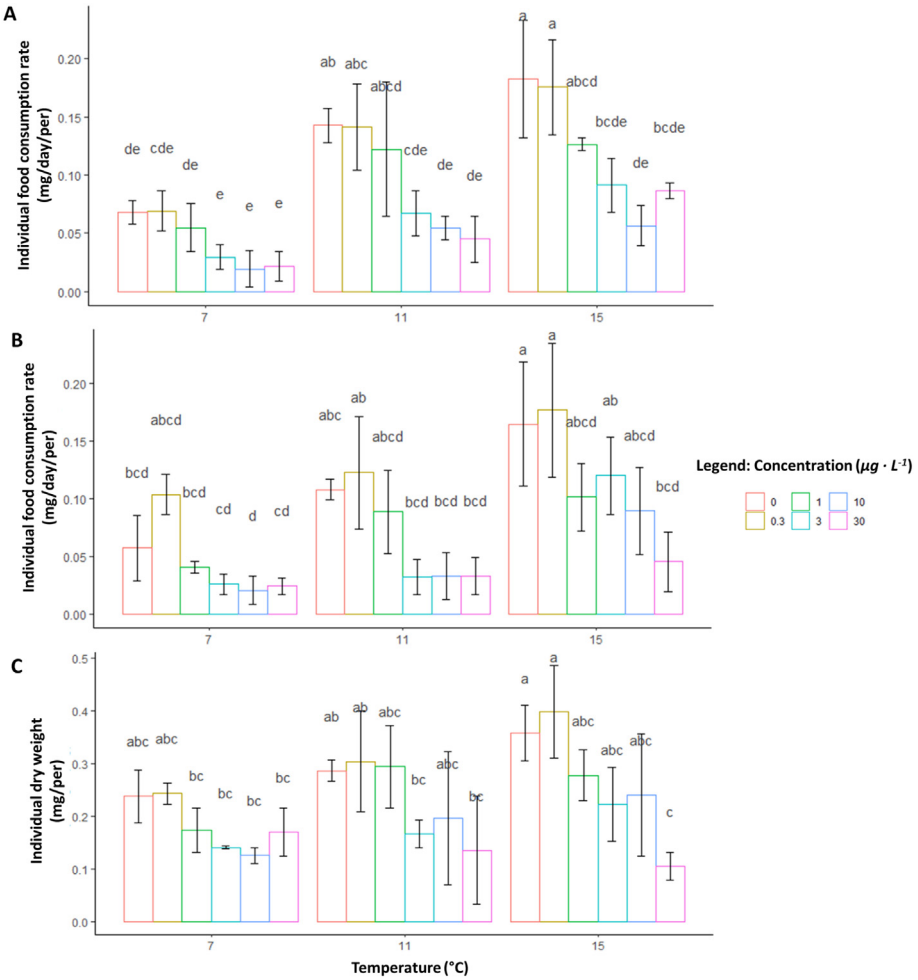

Fig. 4. Food consumption rates per individual in the first two weeks (A) and the last two weeks (B), and the dry weight of *Gammarus pulex* in the IMI experiment (n = 5 for control, n = 3 for treatment groups). Different letters indicate significant differences between clones and treatments, using TukeyHSD ($p < 0.05$). conc = concentrations in $\mu g \cdot L^{-1}$, temperature in °C.

3.3. Sublethal effects of IMI and FPF at different temperatures

3.3.1. The food consumption inhibition

Higher temperatures increased food consumption while IMI and FPF decreased it (Figs. 4 and 5). Furthermore, higher temperatures exacerbated the food consumption inhibition of IMI and FPF (Figs. 4 and 5). For IMI, in the first two weeks, at 15 °C, the food consumption at 1 μg·L^{-1} was significantly lower than in the control group, while it was 3 μg·L^{-1} at 11 °C, and there was no significant difference among concentrations at 7 °C (Fig. 4, A1). However, in the late two weeks, only 30 μg·L^{-1} IMI significantly inhibited food consumption compared to the control at 15 °C (Fig. 4, A2). For FPF, the food inhibition extent was less than for IMI. Only 30 μg·L^{-1} significantly inhibited food consumption compared to the control at 15 °C, and only in the first two weeks (Fig. 5, A1 and A2).

Feeding activity is a sensitive sublethal indicator at the individual level, impairing higher levels such as population, community, or ecosystem (Rinderhagen et al., 2000). The detritivorous activity of *G. pulex* is vital to aquatic systems (Olivier Dangles et al., 2004). The effects of temperature (Nilsson, 1974) and pesticides have been explored separately in previous studies. Inhibition of food consumption by IMI and FPF has been found (Nyman et al., 2013; Agatz et al., 2014; Huang et al., 2022a). It was observed that the feeding rate of *G. pulex* was significantly reduced after continuous exposure to 30 μg·L^{-1} IMI for 4 days and 15 μg·L^{-1} IMI for 14 days at 13 °C (Nyman et al., 2013; Agatz et al., 2014). FPF inhibited the food consumption of *G. pulex* at concentrations higher than 0.3 μg·L^{-1} after 28 days of exposure at 18 °C (Huang et al., 2022a). Overall, our study showed the effect of temperature and IMI and FPF on food consumption and indicated that the interactive effect is higher for IMI than for FPF.

To the best of our knowledge, only a few studies have explored the interactive effects of temperature and chemicals on food consumption. In a recent study by Theys et al. (2020), the food consumption of another freshwater isopod, *Asellus aquaticus,* was measured under exposure to the pesticide chlorpyrifos, combined with increased mean temperature and in the presence or absence of daily temperature fluctuations (DTF) (Theys et al., 2020). They found that organisms' food consumption decreased when exposed to chlorpyrifos and DTF simultaneously.

3.3.2. The influence of temperatures and chemicals on body weight of G. pulex

The dry weight results of *G. pulex* were consistent with the food consumption result (Section 3.3.1; Figs. 4 and 5). Higher temperature enhanced food consumption and increased the dry weight of organisms, whereas IMI and FPF exposure inhibited food consumption and decreased weight. The food consumption inhibition by FPF exposure in the last two weeks was not significant, which is consistent with the fresh weight results (Supplementary data Text S3, Fig. S4A and B). The water content increased with chemical treatment and temperature (Supplementary data Text S3, Fig. S4C and D). These results could be related to the physical characteristic of *G. pulex* as a previous study found that the water content of *G. pulex* was higher under higher temperature conditions (Maazouzi et al., 2011). They explained that the chitin exoskeleton fixes the body volume of crustaceans, and the lost tissue mass, used as a metabolic fuel, must be replaced with water to maintain the same body volume (Maazouzi et al., 2011). In line

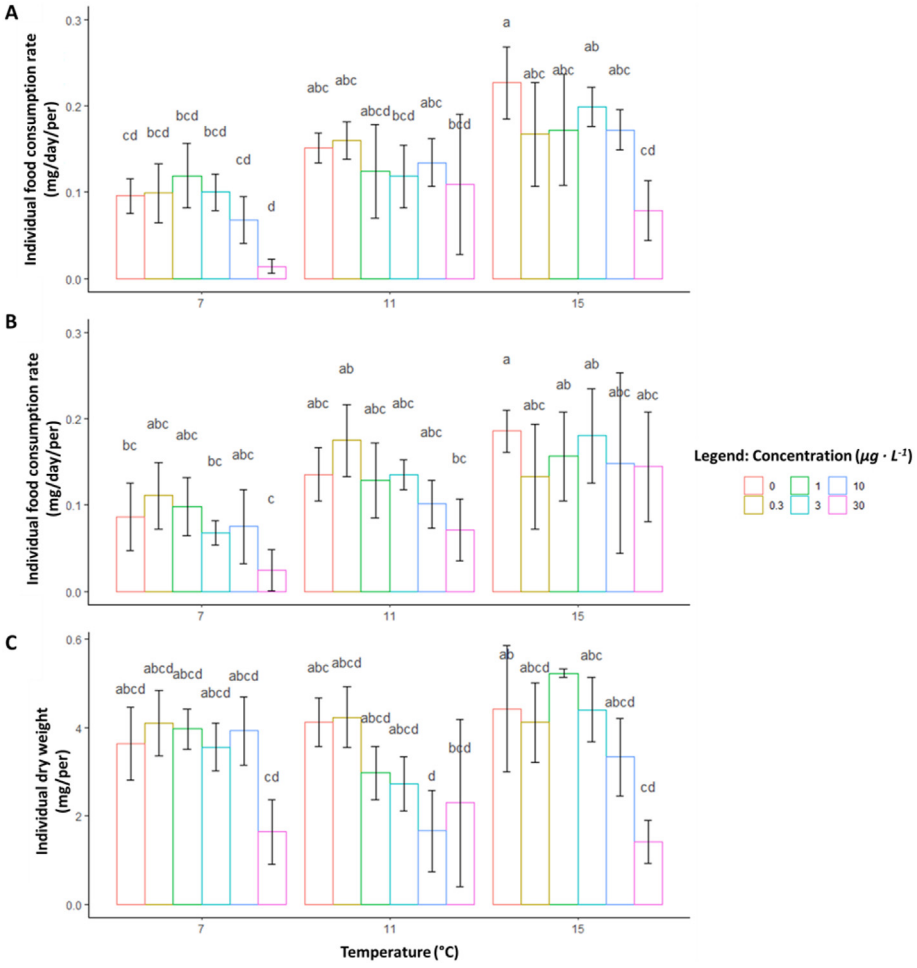

Fig. 5. Food consumption rates per individual in the first two weeks (A) and the last two weeks (B), and the dry weight of *Gammarus pulex* in the FPF experiment (n = 5 for control, n = 3 for treatment groups). Different letters indicate significant differences between clones and treatments, using TukeyHSD ($p < 0.05$). conc = concentrations in μg·L^{-1}, temperature in °C.

with their findings, we also did not find size differences between treatments at each temperature (Supplementary data Text S4).

4. Conclusion

We assessed the effect of temperature on the toxicokinetics and the chronic toxicity of IMI and FPF towards *G. pulex*. For both IMI and FPF, the uptake and elimination rate constants increased with temperature but in different magnitudes. In addition, temperature increased the biotransformation rate of IMI and thus accelerated the generation of the toxic metabolite IMI-ole. Furthermore, we found that higher temperatures increased the toxicity of IMI and FPF over time, where the increase was higher for IMI than for FPF. In addition, the adverse effects of insecticides on sublethal endpoints (i.e., food consumption and dry weight) were exacerbated by elevated temperatures. Overall, our results provided more evidence and understanding of the interaction between increasing temperatures and chemicals' lethal and sublethal effects. Our study indicated the importance of integrating temperature into future toxicity and risk assessments in light of global climate change.

CRediT authorship contribution statement

Anna Huang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Annika Mangold-Döring:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Huitong Guan:** Investigation, Writing – review & editing. **Marie-Claire Boerwinkel:** Investigation, Writing – review & editing. **Dick Belgers:** Investigation, Writing – review & editing. **Andreas Focks:** Conceptualization, Supervision, Writing – review & editing. **Paul J. Van den Brink:** Conceptualization, Resources, Supervision, Writing – review & editing.

Data availability

The raw data is provided in the Mendeley repository https://data.mendeley.com/datasets/6dbgkhzxvx.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158886.

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