Environmental Pollution 293 (2022) 118610

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Toxicokinetics of three insecticides in the female adult solitary bee *Osmia bicornis*

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ARTICLE INFO

Keywords:
Pollinators
Pesticides
Metabolites
Accumulation
Elimination
Degradation

ABSTRACT

The worldwide decline of pollinators is of growing concern and has been related to the use of insecticides. Solitary bees are potentially exposed to many insecticides through contaminated pollen and/or nectar. The kinetics of these compounds in solitary bees is, however, unknown, limiting the use of these important pollinators in pesticide regulations. Here, the toxicokinetics (TK) of chlorpyrifos (as Dursban 480 EC), cypermethrin (Sherpa 100 EC), and acetamiprid (Mospilan 20 SP) was studied for the first time in *Osmia bicornis* females at sublethal concentrations (near LC_{20s}). The TK of the insecticides was analysed in bees continuously exposed to insecticide-contaminated food in the uptake phase followed by feeding with clean food in the decontamination phase. The TK models differed substantially between the insecticides. Acetamiprid followed the classic one-compartment model with gradual accumulation during the uptake phase followed by depuration during the decontamination phase. Cypermethrin accumulated rapidly in the first two days and then its concentration decreased slowly. Chlorpyrifos accumulated similarly rapidly but no substantial depuration was found until the end of the experiment. Our study demonstrates that some insecticides can harm solitary bees when exposed continuously even at trace concentrations in food because of their constant accumulation leading to time-reinforced toxicity.

1. Introduction

The presence of a range of insecticides in pollen and nectar of crop plants has been confirmed in a number of studies (Zioga et al., 2020; Tosi et al., 2018; Sanchez-Bayo and Goka, 2014; Mullin et al., 2010), posing a potential risk to pollinators, either alone or in cumulative action with other natural toxicants (e.g. flavonoids, quercetin, nicotine) present in pollen and nectar. Commonly used insecticides in agriculture that include organophosphates, systemic neonicotinoids (Dai et al., 2017) and pyrethroid formulations (Yang et al., 2019) have been reported to exert toxic effects in honeybee pollinators when consumed orally (Tavares et al., 2019). Even at low concentrations the dietary exposure to insecticides can cause sublethal effects in bees, such as reduction in reproduction (Rundlöf et al., 2015) and abnormal ecological performance (Sgolastra et al., 2019; Tavares et al., 2019). If an organism is exposed continuously for a prolonged time to trace dietary residues, the level of an insecticide and/or its metabolite can build up over time in its tissues and may become lethal or cause non-lethal yet biologically important negative effects (Zaworra et al., 2019; Bednarska et al., 2017). This scenario is plausible in case of wild bees because their adult lifespan and foraging usually overlap with the blooming period of bee-attractive crops, like oil seed rape, usually treated with a range of pesticides (Jauker et al., 2012). Monitoring the kinetics of insecticides in bees is, thus, crucial for understanding their sublethal effects on pollinators. Despite the evidence that oral exposure of wild bee pollinators to different insecticides may result in toxicity, toxicokinetic data for active substance in agrochemical formulations are still lacking, especially for solitary bees. Empirical models of insecticide toxicokinetics in bees may help to (i) understand the time course of toxicity and (ii) bridge the gap between residues detected in bees (and bee products) and threshold toxicity values.

Time-dependent and species-specific insecticide toxicity is influenced by a multitude of species- and compound-specific properties and processes such as accumulation, distribution, biotransformation (breakdown of the active substance into metabolites; detoxification) and elimination – all add up to toxicokinetics (TK). If detoxification of a

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https://doi.org/10.1016/j.envpol.2021.118610
Received 24 May 2021; Received in revised form 12 November 2021; Accepted 28 November 2021
Available online 30 November 2021

toxicant is rapid, its concentration in the organism may equilibrate with a constant accumulation rate at relatively low-level (Rozman et al., 2010) and cause minimal damage to the organism. For instance, orally ingested imidacloprid can be cleared rapidly from animal body, with biological half-life of ~4 h for honeybees (Suchail et al., 2004a) and 10 h for bumble bees (Cresswell et al., 2014). So, theoretically, imidacloprid is unlikely to bioaccumulate in either species during exposures at ecologically relevant timescales that tend to weeks, and consequently should cause minimal fatalities. In contrast, slowly detoxified insecticides accumulate gradually in the organism, eventually exerting time-reinforced effects (Tennekes and Sánchez-Bayo, 2011; Rozman et al., 2010), such as taking longer time to recover to normal biological state. Under equivalent treatment conditions and exposure, insecticides with longer in-organism half-life, that cause time-reinforced toxicity and may up/down regulate certain enzymes (e.g. P450s), pose a greater threat. However, eventually this is the ratio between internal toxicity threshold and the ultimate concentration reached in the organism that determines actual toxic effect. Therefore, toxicological models predicting the rate of elimination of insecticides from organisms, combined with data on internal toxicity thresholds, can be useful in assessing their sublethal and chronic effects. On the other hand, some insecticide metabolites are known to be more toxic than the corresponding parent compounds (Tomizawa and Casida, 2005). Because insecticides can be metabolized in the bee body over time, the parent compounds may no longer be detectable or be detected at very low levels (Suchail et al., 2004b). There are limited number of studies on the kinetics of pesticides in bees (Bonzini et al., 2011; Brunet et al., 2005; Suchail et al., 2004a, 2004b), and mostly confined to honeybees. The very few toxicokinetic data available for metabolites of commonly used pesticides make the results difficult to interpret for toxicity, especially in case of delayed mortality findings. Toxicokinetics data for both the active substances and their major toxicologically relevant metabolites are, thus, necessary to conclude on such delayed effects in bees.

In our previous studies, we tested toxic effects in *Osmia bicornis* upon exposure to three agrochemical formulations (containing chlorpyrifos, cypermethrin and acetamiprid). All three insecticides caused high mortality in freshly emerged females fed with contaminated sucrose solution, in all cases the estimated LC_{50s} at infinite time being lower than concentrations recommended for field-application (Mokkapati et al., 2021). Here, we investigate the toxicokinetics of these three insecticides and their corresponding main metabolites in *O. bicornis* upon continuous oral exposure followed by depuration with feeding the bees uncontaminated food. Based on the measured internal concentrations in bees over time, parameters of one-compartment toxicokinetic model, i.e. assimilation and elimination rate constants, were estimated and compared between the studied agrochemicals.

2. Materials and methods

2.1. Chemicals

Three commercially available agrochemicals used were Mospilan 20 SP (with acetamiprid as the active ingredient, a. i.), Dursban 480 EC (a.i. chlorpyrifos) and Sherpa 100 EC (a.i. cypermethrin). The recommended field application rates (RAR) given by their respective manufacturers (0.12 kg ha^{-1} for Mospilan, 0.60 L ha^{-1} for Dursban and 0.30 L ha^{-1} for Sherpa) and the recommended dilution of the products in 300 L were used to calculate Recommended Application Concentrations (RAC) of the active ingredients (20% acetamiprid for Mospilan, 44.86% chlorpyrifos for Dursban and 10.76% cypermethrin for Sherpa) to prepare experimental solutions with respect to actual concentrations used by farmers in the field. Concentrations close to the 7 day-LC_{20} values estimated by Mokkapati et al. (2021), namely 0.1 RAC for Mospilan 20 SP (i.e. 8 μg mL^{-1} acetamiprid), 0.0001 RAC for Dursban 480 EC (i.e. 0.1 μg mL^{-1} chlorpyrifos) and 0.2 RAC for Sherpa 100 EC (i.e. 20 μg mL^{-1} cypermethrin) were used in this experiment. Contaminated food was prepared as 33% w/w sucrose solution of Mospilan-acetamiprid (ACT treatment), Sherpa-cypermethrin (CYP treatment) and Dursban-chlorpyrifos (CHP treatment).

The analytical standards of chlorpyrifos (CHP), chlorpyrifos-oxon (OXN), acetamiprid (ACT), N-desmethyl acetamiprid (AND), cypermethrin (CYP), rac,cis-permethrinic acid (PAC) and triphenyl phosphate (TPP) were purchased from Sigma-Aldrich (Steinheim, Germany), Dr. Ehrenstrofer (Augsburg, Germany), and Toronto Research Chemical (Toronto, Canada). Stock solutions of standards (2000 μg mL^{-1} each) for chemical analysis were prepared in acetone (GC grade, POCh, Poland) and stored in dark at −20 °C. Intermediate (10 μg mL^{-1}) and working (0.1–5 μg mL^{-1}) standards were prepared in acetone for use on the day of chemical analysis.

2.2. Osmia bicornis emergence and maintenance

In March 2018, cocoons of the solitary bee *O. bicornis* (previously known as *O. rufa* L.) (*Hymenoptera: Megachilidae*) were purchased from the commercial supplier (BioDar, Poland) and stored at 4 °C until use. As *O. bicornis* females solely construct nest cells and collect pollen and nectar for future offspring, while the role of males is limited to insemination (Raw, 1972), only emerged adult females were used in the study. In April 2018, before the planned experiment, ca. 2500 comparatively large cocoons (presumably females) were put in cardboard boxes, (ca. 100 cocoons per box) which were placed in plastic emergence containers (46 × 30 × 17 cm) with air flow provision from the top. Adult bees were emerged from the cocoons in the climatic chamber at 20 ± 2 °C, 70 ± 5% relative humidity (RH) and 16:8 h light:dark (L:D) regime. Each day, males, if any, were removed from the emergence boxes to minimise fertilization, and females were fed *ad libitum* with 33% w/w sucrose solution before used in the experiment.

2.3. Experimental design

The experiment was designed as a typical toxicokinetic study with continuous exposure to a pesticide (contamination phases, also described in the literature as uptake phase) after which the bees were offered uncontaminated food (decontamination phases, in the literature described also as elimination phase). Originally, we planned 10 days of contamination phase and 10 days decontamination phase for all three insecticides, but due to higher mortality (~33%) than expected (~20%) in ACT and CYP treatments, we shortened both phases to 8 days for these two insecticides. Prior to the experiment, bees were starved for 24 h and then the 7–14 days old females were transferred in groups of 14 bees per box, with 55 boxes (14 × 12 × 17 cm disposable plastic box with flat lid with holes for air supply) per insecticide. On the day of treatment, the movement of bees was slowed down by cooling them at 4 °C (no longer than 10 min) for easy handling. The treatment boxes were provided with respective insecticide-contaminated 33% (w/w) sucrose solutions using two slant positioned 2 mL disposable syringes with the needle tips cut-off for easy access to the feeding solution by the bees. Whole experiment was conducted in a walk-in climatic chamber (20 ± 2 °C, 70 ± 5% RH, 16:8 L:D) with daily observation. In order to simulate real-field conditions of continuous food availability, the bees were fed *ad libitum* throughout the experiment by daily re-filling the syringes with their respective treatment solutions which were kept in glass bottles alongside the treatment boxes in the same climatic chamber.

For CHP treatment, bees were collected at day 0 (i.e., before starting the exposure), after 1, 2, 4, 6, 8 and 10 days (contamination phase) and 11, 12, 14 and 20 days (decontamination phase). For ACT and CYP treatments, the days of bee collection were 0, 1, 2, 4, 6, 8 (contamination phase) and 9, 10, 12, 16 (decontamination phase). For each insecticide treatment, on each sampling day, 5 treatment boxes were chosen at random and 10 bees from each box were collected as one sample, placed in cryovials and stored at −80 °C for further chemical analysis (5 sample replicates per treatment per day; each replicate comprised of 10 bees).

Bees were randomized between the boxes of same treatment throughout the experiment and the positions of boxes in the walk-in chamber were changed every day. At the last sampling day, 3 and 4 replicates were available for CYP and ACT treatments, respectively. Besides, 4 replicates were analysed for ACT at day 10 as one sample was lost during storage. The total number of biological samples, each comprised of 10 bees, for the analysis were 48 for ACT and CYP each, and 55 for CHP. Additionally, at each day of bee sampling, samples of contaminated sucrose solutions (50 mL each) were collected and stored at −80 °C for chemical analysis to estimate half-life values (DT_{50}) of the studied insecticides.

Chemical extraction was performed based on QuEChERS method (Anastassiades et al., 2003) using citrate salts as buffering agents (Anastassiades et al., 2007) and the extracted samples were analysed using GC-MS (see Supplementary Materials for details) for ACT, CYP and CHP and their metabolites: N-desmethyl acetamiprid (AND), rac, cis-permethrinic acid (PAC) and chlorpyrifos-oxon (OXN), respectively.

2.4. Chemical analysis of insecticides

Analytical procedures for GC-MS appeared immensely challenging technically because of the very low concentrations and in such small organisms as *O. bicornis*, resulting in concentrations in extracts from single bees far below the detection limits (LOD) and quantification limits (LOQ) for the insecticides and their metabolites. Hence, we used ten bees from each box as one biological sample for the detection of studied insecticides and their metabolites. Wet weight of the sample (10 bees) was measured (Sartorius M2P, Germany) to the nearest 0.001 g.

2.5. Chemical extraction from bees and sucrose solutions

Chemical extraction was performed based on QuEChERS method (Anastassiades et al., 2003) using citrate salts as buffering agents (Anastassiades et al., 2007). Briefly, sample extracts (10 bees per sample weighing ~1 g) were prepared in 7 mL polyethylene ampules by injecting 50 μL triphenyl phosphate as internal standard. For each sample, 1 g of extraction salts were added and homogenized in water-acetonitrile 1:1 mixture (2.5 mL) using bead homogeniser (Omni International, Kennesaw, GA, US) for 1 min. Homogenate/extracts were centrifuged at 1000 g for 10 min and the clear acetonitrile layer was transferred to new test tubes using Pasteur's pipets. These extracts were purified by liquid partitioning and dispersive solid phase extraction (dSPE) using 120 mg of PSA (primary secondary amine) sorbent and 1 min shaking on vortex. After separation, the extracts were placed in new test tubes and evaporated to dry in the gentle stream of nitrogen at 28 °C using RapidVapVertex (LabConco KS, US) evaporator. Each dried residue was dissolved in 0.1 mL acetone (POCh, Poland) and transferred to GC vials for further analysis.

Similar extraction procedure as above was followed for the determination of parent compounds (ACT, CHP and CYP) in feeding sucrose solutions, using a different dSPE sorbent (combination of 100 mg PSA + 100 mg octadecylsilyl (C18)) in a single clean-up step (Vázquez et al., 2015).

2.6. GC-MS analysis for the determination of pesticide concentrations in extracted samples

Measurements of ACT, CHP and CYP and one of their main metabolites (N-desmethyl acetamiprid (AND) (Brunet et al., 2005), chlorpyrifos-oxon (OXN) (Poquet et al., 2016), and rac,cis–permethrinic acid (PAC) (Knaak et al., 2012), respectively for ACT, CHP and CYP) along with the internal standard triphenyl phosphate (TPP) were carried out using liquid-gas chromatograph, PerkinElmer Clarus 600 GC system coupled with quadrupole mass spectrometer Clarus 600C (on-column injector; fused high temperature silica column, 60 m × 0.25 mm internal dimension, i. d.; 5% phenyl/95% methyl polysiloxane equivalent phase, 1 μm film thickness; protected by 5 m × 0.53 mm i. d. inert pre-column). Helium gas was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. For each sample, 4 μL of extract was injected at split ratio 1:50 into the chromatograph inlet at 50 °C initial temperature. After 1 min, inlet temperature was immediately increased to 310 °C. Oven temperature after injection was programmed to raise from 70 °C to 295 °C at a constant rate of 10 °C per min and held for 12.5 min. Quantification was done using mass detector in selected ion monitoring (SIM) mode, registering characteristic base peak at given m/z values for each ion (314 m/z – CHP; 126 m/z – ACT; 163 m/z –CYP). The limits of detection (LOD) values were calculated based on the values of standard deviation of response from calibration regression statistics and are given in Table S1 for ACT, CYP and CHP in sucrose solution (μg mL^{-1}) and bee samples (μg g^{-1}). The LOD values for N-desmethyl acetamiprid (AND), rac,cis–permethrinic acid (PAC) and chlorpyrifos-oxon (OXN) in bee samples were 0.001, 0.009 and 0.002 μg g^{-1}, respectively.

To check the recovery of the studied compounds, both sucrose solution (1 mL per sample) and bees (5 samples each consists of 10 female *O. bicornis* bees) were spiked with 100 μL of analyte solution separately for each insecticide to determine their respective known concentration in extracts. These samples were processed according to the above-mentioned procedure and the recovery was 0.92 ± 0.014 (average of 4 samples ±SE) for ACT, 0.93 ± 0.025 for CYP and 0.87 ± 0.019 for CHP in sucrose solution and 0.95 ± 0.009, 0.90 ± 0.01 and 0.92 ± 0.007, respectively in bee samples. The recovery of N-desmethyl acetamiprid (AND), rac,cis–permethrinic acid (PAC) and chlorpyrifos-oxon (OXN) in bee samples was 0.97 ± 0.01, 0.88 ± 0.013 and 0.88 ± 0.006, respectively. The analytical results for the insecticide-exposed experimental bees were not corrected for recovery.

3. Data analysis

Degradation kinetics of insecticides in feeding solutions was described using the non-linear first-order kinetics.

$$C_t = C_o \cdot e^{-k \cdot t}$$

where C_t represents the concentration of an active ingredient in the feeding sucrose solution at time t, C_o is the initial concentration in sucrose solution at day 0, and k – the degradation constant. The half-life values (DT_{50}) of the pesticides were calculated as. $DT_{50} = \ln(2)/k$

Toxicokinetics (TK) of the studied insecticides was analysed using the one-compartment first-order model (Skip et al., 2014), with t_c indicating the time of switching bees to uncontaminated food (switch point from the uptake to decontamination phase):

For the accumulation phase ($t \leq t_c$):

$$C_I(t) = C_{Io} \cdot e^{k_E \cdot t} + C_{Eu}\frac{k_A}{k_E}\left(1 - e^{-k_E \cdot t}\right)$$

and for the depuration phase ($t > t_c$):

$$C_I(t) = C_{It_c} \cdot e^{k_E \cdot (t - t_c)} + C_{Ed}\frac{k_A}{k_E}\left(1 - e^{-k_E \cdot (t - t_c)}\right)$$

where

$$C_{It_c} = C_o \cdot e^{-k_E \cdot t_c} + C_{Eu}\frac{k_A}{k_E}\left(1 - e^{-k_E \cdot t_c}\right)$$

where C_I – internal toxicant concentration at time t (nmol g^{-1} bee body mass); C_{Io} – internal toxicant concentration at t = 0 (nmol g^{-1}); C_{Eu}, C_{Ed} – measured exposure concentration in feeding solution (nmol mL^{-1}) during the uptake (= contamination) and decontamination phases respectively; k_A – assimilation rate constant (day^{-1}); k_E – elimination rate constant (day^{-1}). The model was fitted to the data using the Marquardt method.

Data were analysed by fitting the TK model to the sum of parent compound and its main metabolite (as nmol g^{-1} bee body mass) after

removing unusual outliers (Studentized residual < -3 or >3), if any. In case of CHP and CYP, since the classic model did not fit well ($R^2 \approx 0$ or apparent deviation of the model from data was visible), we additionally fitted a modified model, in which instead of a fixed switching point set to the last day of the contamination phase (t_c), a breakpoint day (B) was estimated from the best-fit model. Although such an estimated breakpoint cannot be interpreted clearly from the point of view of the classic TK model, it indicates that concentrations of some insecticides may start decreasing even if bees are still exposed to pesticide-contaminated food. The kinetics parameters were checked for significance using asymptotic 95% confidence intervals. The effect of time on average mass of bee samples (log_{10}-transformed data) was checked using ANOVA (day 0 samples were excluded to avoid initial 24 h pre-starvation effect on sample mass). Data analysis was performed using Statgraphics Centurion XVIII version 18.1.06.

4. Results

The percent measured concentrations of active ingredients in the initial feeding sucrose solutions (i.e. day 0) were 93%, 94% and 76% of the nominal concentrations for ACT, CYP and CHP, respectively. The degradation of insecticides in time was relatively better described for CHP and CYP ($R^2 \geq 65\%$) compared to ACT ($R^2 = 31\%$), with DT_{50} estimated at 97 days for ACT, 27 days for CHP and 95 days for CYP and (Table S1).

Significant effect of time on the bee body mass was found in ACT ($p = 0.0001$) and CYP ($p = 0.035$) treatments, but not in CHP treatment ($p = 0.6$). The analysis done separately for each of TK phases indicated no effect of sampling day on mean body mass in the contamination phase ($p \geq 0.6$ for both ACT and CYP), but the body mass decreased over time in the decontamination phase ($p \leq 0.017$; Table S2).

4.1. Toxicokinetics of ACT and its metabolite AND

Traces of ACT (0.045 ± 0.035 nmol g^{-1}; mean ± standard deviation, SD) were found in the bees before starting the experiment (day 0). While fitting the TK model for the combined concentrations of ACT and its metabolite (AND) in bees, one unusual outlier (Studentized residual < -3) was detected at day 8 and excluded from the model ($n = 47$). The final model was well fitted with $R^2 = 79.1\%$ ($R^2_{adj} = 78.6$) and significant coefficients of assimilation $k_A = 0.0345$ day^{-1} (95% CI 0.0266–0.0424) and elimination $k_E = 0.343$ day^{-1} (95% CI 0.254–0.432) (Table 1). The internal ACT and AND concentrations increased asymptotically during the accumulation phase with a rapid decrease in the elimination phase (Fig. 1a). Mean (±SD) internal toxicant concentrations reached the highest level for ACT (0.91 ± 0.12 nmol g^{-1}) and AND (2.76 ± 0.57 nmol g^{-1}) on the 6th day of the exposure. After feeding the bees with uncontaminated food during the decontamination phase, the internal concentrations decreased rapidly to 0.27 ± 0.14 nmol ACT g^{-1} and 0.36 ± 0.25 nmol AND g^{-1} by the end of the experiment (i.e. day 16) (Fig. 1b–c).

4.2. Toxicokinetics of CYP and PAC

No CYP was detected in the samples of *O. bicornis* at day 0. For CYP and its metabolite PAC, after removing one unusual Studentized residual (>3), the model was fitted with $R^2 = 17.1\%$ ($R^2_{adj} = 15.2\%$; $n = 47$) and both TK parameters were significant: $k_A = 0.0078$ day^{-1} (95% CI 0.0041–0.0114) and $k_E = 0.659$ day^{-1} (95% CI 0.333–0.985) (Table 1). Although the model was significant, it did not explain well the very fast initial concentration increase (maximum concentrations reached already at day 2) followed by a slow decrease in concentration thereafter (Fig. 2a). By day 2, the mean (±SD) internal concentrations reached the highest levels for both CYP (0.36 ± 0.106 nmol g^{-1}) and PAC (0.34 ± 0.188 nmol g^{-1}) and then decreased gradually reaching 0.08 ± 0.021 nmol CYP g^{-1} (Figs. 2c) and 0.05 ± 0.018 nmol PAC g^{-1} (Fig. 2d) towards the end the of the experiment (i.e. day 16).

The modified model with estimated breakpoint *B*, after excluding two data points with Studentized residuals >3 ($n = 46$), explained 65.7% (R^2) of the total variance ($R^2_{adj} = 64.1\%$), with $k_A = 0.0101$ day^{-1} (95% CI 0.0074–0.0127) and $k_E = 0.103$ day^{-1} (95% CI 0.068–0.137). The breakpoint *B*, that is time when maximum concentration was reached and started to decrease, was estimated as 1.47 days (95% CI 1.04–1.90) (Table 1). These results indicate that indeed a very fast accumulation of CYP + PAC occurred in the body (with estimated maximum level between day 1 and 2) followed by a slow decrease of concentrations of both the parent compound and its metabolite (Fig. 2b).

4.3. Toxicokinetics of CHP and OXN

No CHP was detected in *O. bicornis* at day 0. The internal concentrations of chlorpyrifos (CHP) together with its main metabolite chlorpyrifos-oxon (OXN) revealed very high variance throughout the experiment. The classic model basically did not explain the TK pattern ($R^2 = 0.0\%$), even if kinetic coefficients were significant: $k_A = 0.556$ day^{-1} (95% CI 0.251–0.861), $k_E = 0.232$ day^{-1} (95% CI 0.075–0.389) (Table 1). No outliers were detected in this case ($n = 55$). Similarly to CYP kinetics, the model did not catch the fast initial increase in concentration at the very beginning of the accumulation phase. Additionally, virtually no elimination until the end of the experiment was observed (Fig. 3a). Besides, CHP concentrations (Fig. 3c) were at least one order of magnitude lower than OXN concentrations (Fig. 3d) since the 1st day of the experiment, indicating the rapid metabolism of CHP into OXN in the bees. However, the measured concentrations of both

Table 1
Toxicokinetics of insecticides in the female adult solitary bees *Osmia bicornis* upon oral exposure to sublethal concentrations of three insecticide-based agrochemicals: Mospilan 20 SP (a.i. acetamiprid - ACT and its metabolite acetamiprid N-desmethyl - AND), Sherpa 100 EC (a.i. cypermethrin – CYP and its metabolite rac-cis-permethrinic acid - PAC) and Dursban 480 EC (a.i. chlorpyrifos – CHP and its metabolite chlorpyrifos-oxon - OXN).

Treatment chemicals detected	C_E (nmol L^{-1})	C_{Io} (nmol g^{-1} sample)	Model*	N	Estimated kinetics parameters (95% CI lower - upper)			R^2	R^2_{adj}
					k_A (nmol g^{-1} day^{-1})	k_E (day^{-1})	B (days)		
ACT + AND	36	0.045	Classic	47	0.0345 (0.0266–0.0424)	0.342 (0.251–0.434)		79.1	78.6
CYP + PAC	48	0	Classic	47	0.0078 (0.0041–0.0114)	0.659 (0.333–0.985)		17.1	15.2
			with estimated B	46	0.0101 (0.0074–0.0127)	0.103 (0.068–0.137)	1.47 (1.04–1.90)	65.7	64.1
CHP + OXN	0.29	0	Classic	55	0.556 (0.251–0.861)	0.232 (0.075–0.389)		0.0	0.0
			with estimated B	55	0.936 (0.526–1.347)	0.045 (−0.004 - 0.094)	2.47 (1.12–3.83)	10.4	6.9

Note: Model parameters estimated based on nominal concentrations of a.i. in the feeding solution (C_E) and initial internal a.i. concentration in bees (C_{Io}); N – sample size, k_A – assimilation rate constant, k_E – elimination rate constants, B – day at which bees started decontamination. Kinetics parameters were estimated by fitting a one-compartment model to data including both contamination and decontamination phases of the experiment.

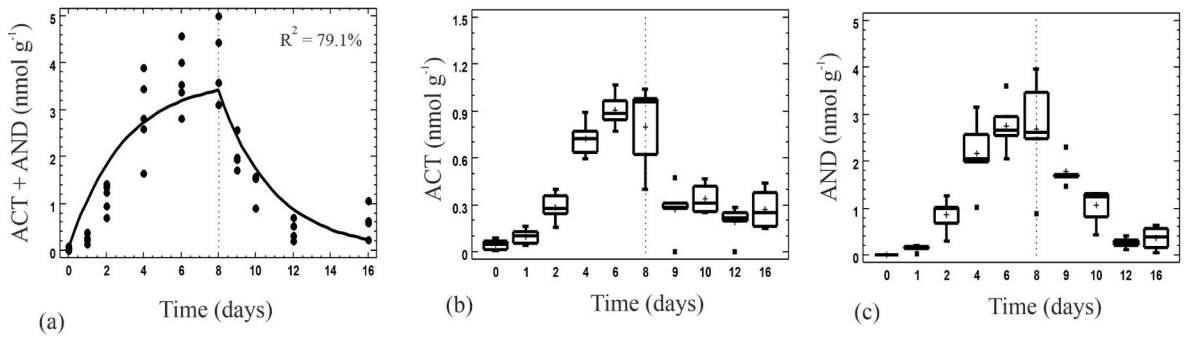

Fig. 1. (a) Accumulation and elimination kinetics of the sum of acetamiprid (ACT) and its metabolite acetamiprid N-desmethyl (AND) in the female adult solitary bees *Osmia bicornis* exposed orally to sublethal concentration of Mospilan 20 SP (a.i. ACT). Solid dots represent measured internal concentrations in samples (10 bees each). Dotted vertical line indicates the start day of feeding bees with uncontaminated sucrose solution (decontamination phase). Solid line shows the estimated one-compartment model fitted simultaneously to contamination and decontamination phases, using the nominal concentration of ACT in the feeding solution; see Table 1 for the estimated model parameters. (b) and (c) – measured concentrations of ACT and AND in the bees during the experiment. Midline represents the median and plus (+) indicates arithmetic sample mean, outside edges of the box mark the 25th and 75th percentile, and the whiskers extend to the minimum and maximum values except for outliers which are marked as solid squares.

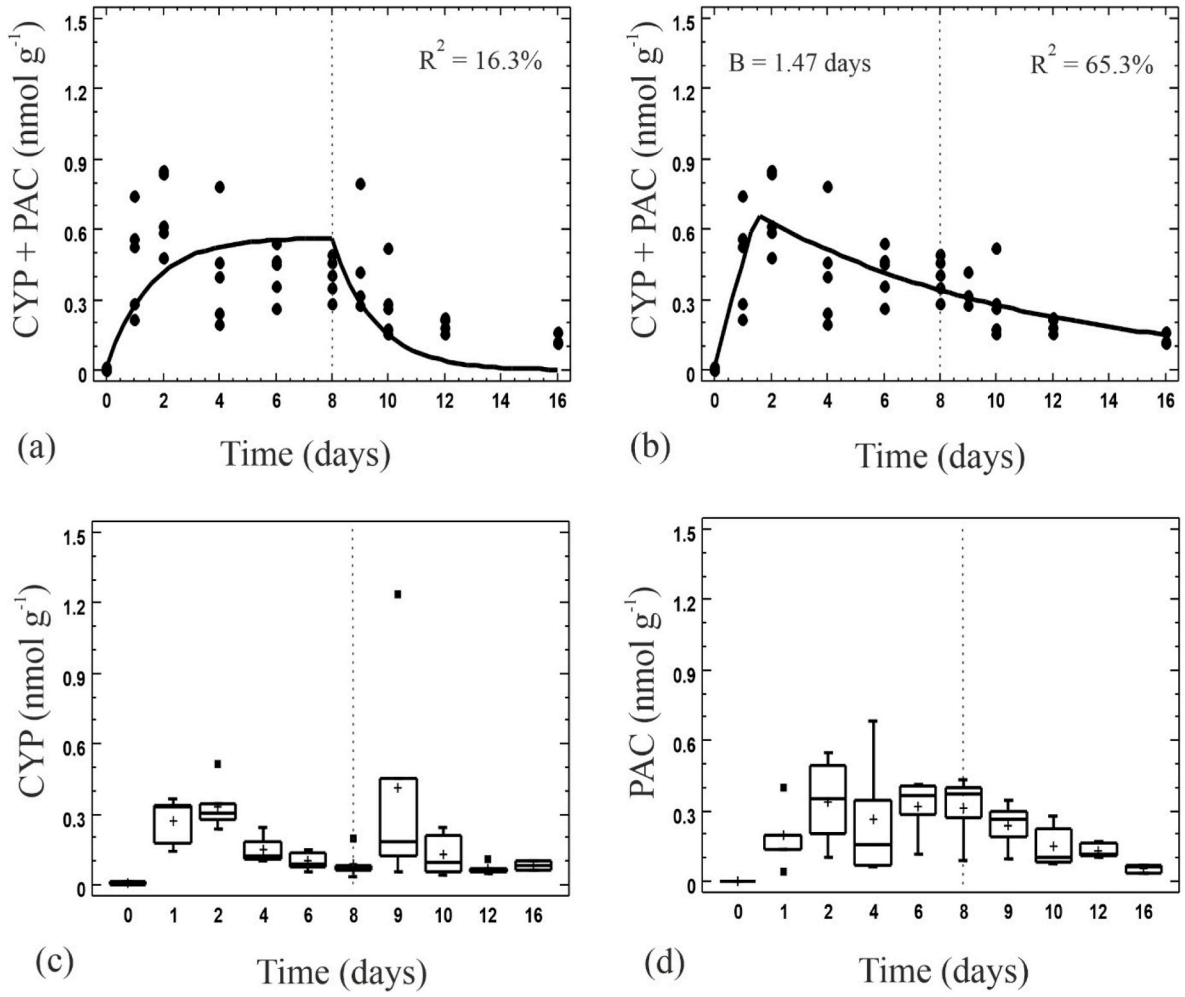

Fig. 2. Accumulation and elimination kinetics of the sum of cypermethrin (CYP) and its metabolite rac-cis-permethrinic acid (PAC) in the female adult solitary bees *Osmia bicornis* exposed orally to sublethal concentration of Sherpa 100 EC (a.i. CYP). Solid dots represent measured internal concentrations in samples (10 bees each). Dotted vertical line indicates the start day of feeding bees with uncontaminated sucrose solution (decontamination phase). Solid line shows the estimated model fitted simultaneously to contamination and decontamination phases, using the nominal concentration of CYP in the feeding solution: (a) – classic one-compartment model with elimination starting at the day of changing the food to uncontaminated; (b) – one-compartment model with estimated start of depuration (B) (see Table 1 for the estimated model parameters). (c) and (d) – measured concentrations of CYP and PAC in the bees during the experiment. Midline represents the median and plus (+) indicates arithmetic sample mean, outside edges of the box mark the 25th and 75th percentile, and the whiskers extend to the minimum and maximum values except for outliers which are marked as solid squares.

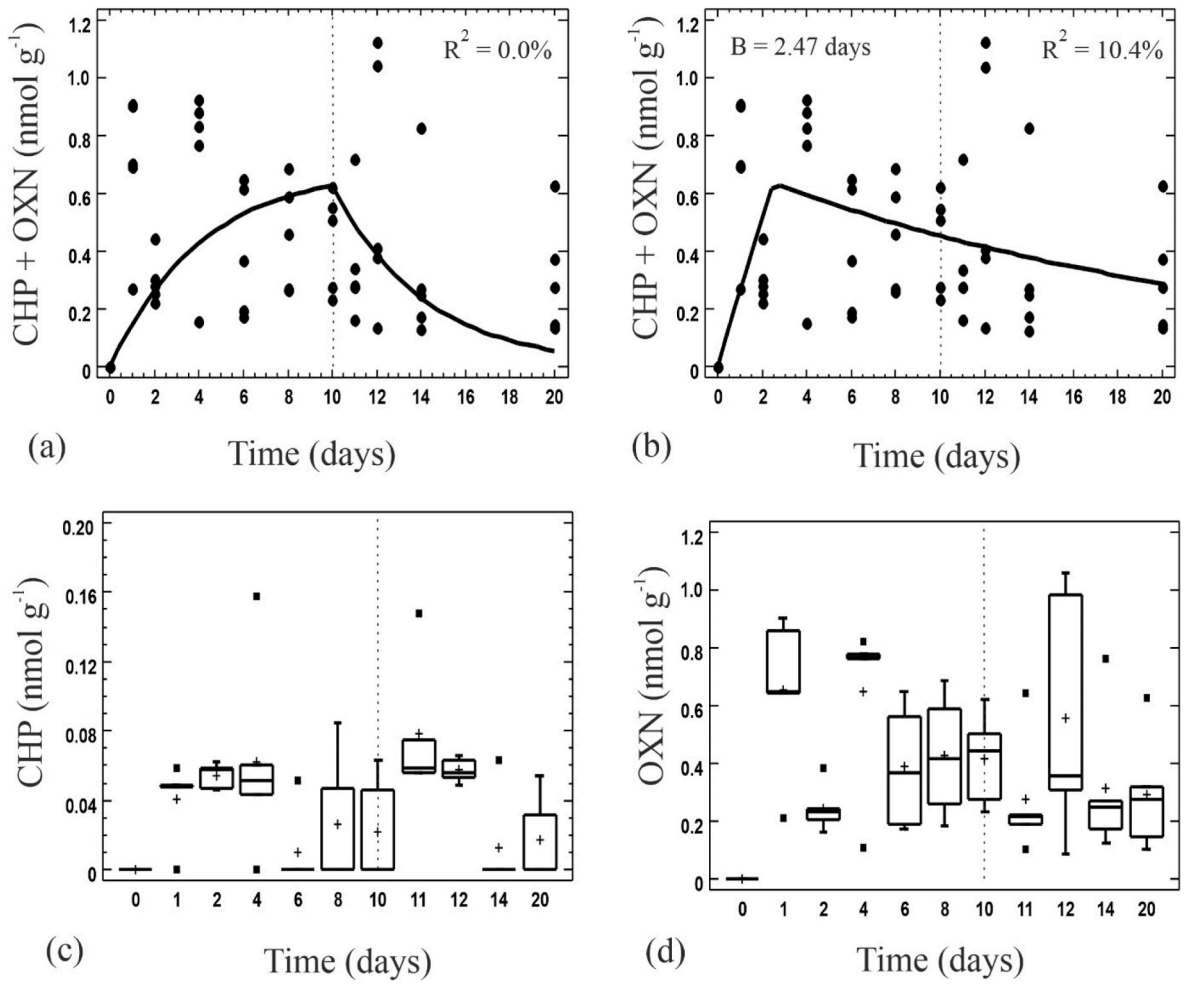

Fig. 3. Accumulation and elimination kinetics of the sum of chlorpyrifos (CHP) and its metabolite chlorpyrifos-oxon (OXN) in the female adult solitary bees *Osmia bicornis* exposed orally to sublethal concentration of Dursban 480 EC (a.i. CHP). Solid dots represent measured internal concentrations in samples (10 bees each). Dotted vertical line indicates the start day of feeding bees with uncontaminated sucrose solution (decontamination phase). Solid line shows the estimated model fitted simultaneously to contamination and decontamination phases, using the nominal concentration of CHP in the feeding solution: (a) – classic one-compartment model with elimination starting at the day of changing the food to uncontaminated; (b) – one-compartment model with estimated start of depuration (B) (see Table 1 for the estimated model parameters). (c) and (d) – measured concentrations of CHP and OXN in the bees during the experiment. Midline represents the median and plus (+) indicates arithmetic sample mean, outside edges of the box mark the 25th and 75th percentile, and the whiskers extend to the minimum and maximum values except for outliers which are marked as solid squares.

CHP and OXN were highly variable even within the same day of sampling (coefficient of variation >30%) (Fig. 3c–d).

With estimated breakpoint (*B*), the resulted model was slightly improved, with $R^2 = 10.4\%$ ($R^2_{adj} = 6.9\%$), no outliers (n = 55) and the breakpoint *B* estimated at 2.47 days (95% CI 1.12–3.83), indicating that the internal CHP + OXN concentrations increased very fast at the beginning of exposure, reaching maximum between day 1 and day 4. In result, the accumulation rate was high (k_A = 0.936 day^{-1}; 95% CI 0.526–1.347) but, in contrast to CYP + PAC, there was virtually no depuration of CHP + OXN as shown by the low and non-significant k_E value (0.045 day^{-1}; 95% CI -0.004 - 0.094) (Table 1). This can be seen on the graph as high concentrations of CHP + OXN even at day 20, i.e. the last day of the experiment (Fig. 3b).

5. Discussion

Based on available experimental DT_{50}s for a range of active substances (81 chemicals) for a broad spectrum of plant materials (grass, cereals, forage crops, cotton, vegetables, tobacco, and foliage of fruit trees), a default residue decline half-life (DT_{50}) of 10 days is used for plant-based food items (EFSA, European Food Safety Authority, 2009). However, for a more accurate and realistic assessment of the exposure over time, EFSA recommended to use the DT_{50}s for the specific test substance in food if available, especially for pesticides more persistent in the environment (>10 days) (EFSA, European Food Safety Authority, 2009). In our experiment, we measured concentrations of the tested insecticides in feeding solutions at different sampling days to assess actual degradation rates in order to include this information in the toxicokinetic models, if necessary. The estimated DT_{50}s in the feeding solutions appeared high enough to assume approximately constant exposure concentration during the uptake phase, allowing us to use the regular TK model. The degradation rates were within the previously reported DT_{50}s for ACT and CHP in soil (2.6–133 days and 19.8–1000 days, respectively) (Krupke and Long, 2015) and in water (13–420 days and 29.6–53.5 days, respectively) (Lewis et al., 2016), but longer that those for CYP (2.4–58.3 days in soil and 3–17 days in water (Lewis et al., 2016)). However, the degradation of pesticides depends on several factors such as solubility, temperature, microbial activity, UV radiation, etc. (Ebeling, 1963; Van Den Heever et al., 2015), all of which could contribute to the observed relatively slow degradation of all three insecticides in the feeding solutions, especially for CYP, when stored in the laboratory in falcon tubes (Keith and Walker, 1992). Since the estimated

DT_{50}s were much longer than the contamination phase, degradation of the insecticides in treatment solutions were not included in the toxicokinetics model. It was also justified by the observed pattern of toxicokinetics discussed in detail below for each insecticide.

Acetamiprid (ACT) metabolises in the bee body through different pathways, involving IM-2-1, IM-1-2, IM-1-3, IM-1-4, IM-0 and IC-0 (Brunet et al., 2005). When ingested orally, ACT is rapidly distributed throughout the organism (half-life $\simeq$ 25 min) and undergoes N-demethylation yielding primarily IM-2-1 (referred to as AND in our study) (Brunet et al., 2005). Because of this rapid metabolism, the toxicity of ACT to bees is often associated with its metabolites, including AND, albeit the metabolites are less toxic than acetamiprid (Zaworra et al., 2019; Brunet et al., 2005). The EU has explicitly established Maximum Residue Level (MRL) of acetamiprid while including its metabolite IM-2-1 (or AND), referring to the sum both compounds (European Union Pesticide Database, 2019). In our previous acute oral test with continuous exposure, Mospilan-ACT was found to be less toxic to *O. bicornis* females (24 h LC_{50} = 18.1 μg a. i. bee^{-1} (considering average consumption of 59.8 μL per day (Sgolastra et al., 2019)) (Mokkapati et al., 2021) than to *A. mellifera* (24 h LC_{50} = 1.69 μg bee^{-1} (Badawy et al., 2015)). However, El Hassani et al. (2008) observed that honeybees fed sucrose contaminated with acetamiprid at a sublethal dose 1 μg bee^{-1} showed increased sensitivity to antennal stimulation, and at the dose 0.1 μg bee^{-1} the long-term retention of olfactory learning was impaired. In addition, 6-chloronicotinic acid (IC-0), a metabolic product of acetamiprid (and imidacloprid), was found to be toxic to honeybees, resulting in 50% mortality within 8 days upon chronic oral exposure at all tested concentrations, from 0.1 to 10 μg L^{-1}, in sucrose solution (Suchail et al., 2001). In this sense, the prolonged intake of acetamiprid results in accumulation of toxicants which may become detrimental to bees. This notion is supported by the observed increase of ACT + AND concentrations in *O. bicornis* throughout the 8-day exposure, as confirmed by the good fit (R^2 = 79%) of the toxicokinetic model. Brunet et al. (2005) found that in orally exposed *A. mellifera* with 100 μg kg^{-1} [14C]-acetamiprid, only 40% of the total radioactivity was eliminated even after 3 days, suggesting that acetamiprid and its metabolites tended to persist in the honeybee bodies. It is also noteworthy that during the exposure phase for ACT (as well as for CYP treatments), higher mortality of bees (33%) was observed than expected with near-LC_{20} concentration exposure. This might be caused by the fact that the bees used in the experiment contained from the very beginning traces of ACT and their metabolic activity could be impaired due to the cumulative action of the insecticides. In *O. bicornis*, we observed that 75% ACT + AND was eliminated from the bee bodies after 2–4 days of the decontamination phase (i.e., 2–4 days since insecticide exposure ceased) and about 82% was eliminated after 8 days of decontamination. The excretion of AND (34%) was comparatively lower than ACT (66%) after 24 h from the start of decontamination phase but it has to be taken into account that with chronic exposure, as in our experiment, AND was still formed by the breakdown of ACT during the decontamination phase.

The metabolism of cypermethrin (CYP) involves a wide range of pathways, primarily by ester cleavage and hydroxylation yielding carboxylic acids that include permethrinic acid (PAC) (Knaak et al., 2012). PAC is a potent insecticide itself, and in fact many permethrinic acid ethyl ester derivatives reveal insecticidal activity (Mirzabekova et al., 2008). In animals, CYP is reported to be metabolically stable, but can be excreted rapidly from the body primarily intact (Leahey, 1985). In *O. bicornis*, the toxicokinetics of CYP and PAC showed (1) the relative stability of CYP and its intact excretion as indicated by the almost equal concentrations of both CYP and PAC till the end of the experiment and (2) the rapid start of excretion of CYP and PAC as illustrated by the poor fit of classic one compartment model with the start of elimination set at day 8 (day of changing the food to uncontaminated) and clearly better fit of the model with estimated start of depuration within ca. 2 days after starting the exposure. It was observed, however, that the bees fed with Sherpa-CYP contaminated food mostly did not show active physical movement (walking, flying) or were even temporarily knocked-down, which might lower their overall food consumption and explain the better fit of estimated breakpoint B toxicokinetic model than the classic one. A severe effect of CYP on bee mobility is in accordance with its mechanism of action by blocking the voltage gated sodium channels in the nervous system leading to paralysis (Kadala et al., 2019; Casida and Durkin, 2013). The decreased consumption could also result from the repellent action of other ingredients/adjuvants of Sherpa, including the strong odour of this agrochemical (Mullin et al., 2015). Similar effects on behaviour and food consumption was observed in *A. mellifera* fed with food containing pyrethroids (Havstad et al., 2019; Oliver et al., 2015; Thompson, 2003). The phenomena discussed above could lead to misinterpretation of the TK model, so its parameters should be treated with caution. On the other hand, the mean body mass of bees in the contamination phase did not decrease during the exposure to Sherpa-CYP, which indirectly indicates that bees did not limit food consumption significantly. However, even if the food consumption was lower in CYP-treated bees, concentrations of CYP and PAC in *O. bicornis* increased in the contamination phase and remained elevated even after 8 days of decontamination. This further highlights the importance of considering toxicokinetics, preferably coupled with toxicity of insecticides and their metabolites if identified, in risk assessment.

Chlorpyrifos (CHP) enters cells by diffusion and readily undergoes oxidative desulfuration, forming chlorpyrifos-oxon (OXN). In our studies a very fast transformation of CHP into OXN was indeed observed in *O. bicornis*, as shown by about one order of magnitude higher concentration of the metabolite than the parent compound already within 24 h from the start of exposure. The very low exposure concentration of CHP might have resulted in very high variance between bee samples observed for both CHP and OXN. Consequently, the data barely allowed to fit a toxicokinetic model for CHP + OXN, with R^2 = 0 for the classic model and R^2 = 10.4% for the model with estimated starting day of the depuration (B). The latter model showed a rapid accumulation of CHP + OXN, reaching peak combined concentration between 1st and 4th day of exposure, followed by almost no excretion of CHP and OXN until the end of experiment, as shown by the elimination rate constant (k_E) not significantly different from 0 (95% CI -0.004 - 0.094) and clearly elevated concentrations of both the parent compound and its metabolite even after 10 days of feeding on uncontaminated food. Rubach et al. (2010) also observed extremely slow elimination of [^{14}C]chlorpyrifos in some freshwater arthropod species. The toxicity of CHP typically is attributed to its oxon metabolite (OXN) which causes more acute lethality by inhibiting acetylcholinesterase more strongly than the parent compound (Poquet et al., 2016). Dahlgren (2014) observed that after 24 h contact exposure, OXN exerted 2-fold greater toxicity than CHP in both workers and queens of honeybees. The very slow or virtually no elimination of CHP + OXN even after 10 days of feeding on the clean food, as observed in our study, indicates high long-term persistence of this insecticide and its most important metabolite, which may cause delayed toxic effects in bees.

Toxicokinetic parameters, when coupled with toxicodynamics, may provide the information necessary for predicting effects of insecticides in bees, leading to improved risk assessment (Panizzi et al., 2017). However, such long-term laboratory ecotoxicological experiments are laborious and expensive, and especially measuring internal concentrations of insecticides and their metabolites at very low concentrations is time consuming, costly and requires high expertise. Knowing toxicokinetic parameters of individual insecticides on the one hand and their effects on the other, may allow to predict effects of field sprays without the necessity for repeating such detailed studies each time in the future, if concentrations of pesticides in nectar and/or pollen is known (measured or calculated). We believe, thus, that toxicokinetic studies can be an important tool in assessment of pesticide effects on pollinators and, in consequence, in regulations of pesticide use.

Because we used the formulation actually applied by farmers, the

patterns observed in this study should better reflect what may actually happen to bees in the field conditions than in case of studies using active ingredients only. On the other hand, laboratory-based studies will always have serious limitations regarding extrapolating their results to field conditions. Among those most import in our study are: constant environmental conditions, easy food availability, no possibility to search for alternative food resources, greatly limited mobility of bees, etc.

6. Conclusions

The TK models for the tested insecticides explained their accumulation in bees during chronic exposure to contaminated food and clearly showed the differences in their kinetics in *O. bicornis*. The comparison of concentrations of parent compounds and their metabolites revealed, in turn, differences in metabolism of the tested chemicals. The estimated TK parameters are important for further studies towards toxicodynamics (TD) for full understanding of the toxic effects induced by insecticides and organismal recovery. If the assimilation and elimination rate constants are known for an insecticide, the time course of toxicant effects and organism recovery can be simulated to assess how certain physiological and biochemical processes vary within and across species (Ashauer et al., 2007). In this study we also showed that at least for some insecticides (here CYP and CHP) the classic one compartment toxicokinetic model may not be adequate.

Credit author statement

Jaya Sravanthi Mokkapati: Conceptualization, Methodology, Data acquisition, Formal analysis, Writing – original draft preparation and editing. **Maciej Choczyński**: Data acquisition, Formal analysis, Writing – original draft. **Agnieszka Bednarska**: Conceptualization, Funding acquisition, Project administration, Supervision, Formal analysis, Writing – review & editing, and **Ryszard Laskowski**: Conceptualization, Funding acquisition, Project administration, Supervision, Formal analysis, Writing – review & editing.

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.

Acknowledgements

We thank Renata Śliwińska and Ligia Kuriańska-Piątek for their assistance in laboratory work. This research was funded by the National Science Centre, Poland (Grant no: 2015/19/B/NZ8/01939). Infrastructure facilities and resources were provided by the Faculty of Biology, Jagiellonian University, Poland (Grant no: DS/WBiNoZ/INoS/756/2018).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118610.

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