



Modelling the effects of the pyrethroid insecticide cypermethrin on the life cycle of the soil dwelling annelid *Enchytraeus crypticus*, an original experimental design to calibrate a DEB-TKTD model

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ABSTRACT

The Dynamic Energy Budget theory (DEB) enables ecotoxicologists to model the effects of chemical stressors on organism life cycles through the coupling of toxicokinetic-toxicodynamic (TK-TD) models. While good progress has been made in the application of DEB-TKTD models for aquatic organisms, applications for soil fauna are scarce, due to the lack of dedicated experimental designs suitable for collecting the required time series effect data. Enchytraeids (Annelida: Clitellata) are model organisms in soil ecology and ecotoxicology. They are recognised as indicators of biological activity in soil, and chemical stress in terrestrial ecosystems. Despite this, the application of DEB-TKTD models to investigate the impact of chemicals has not yet been tested on this family. Here we assessed the impact of the pyrethroid insecticide cypermethrin on the life cycle of *Enchytraeus crypticus*. We developed an original experimental design to collect the data required for the calibration of a DEB-TKTD model for this species. *E. crypticus* presented a slow initial growth phase that has been successfully simulated with the addition of a size-dependent food limitation for juveniles in the DEB model. The DEB-TKTD model simulations successfully agreed with the data for all endpoints and treatments over time. The highlighted physiological mode of action (pMoA) for cypermethrin was an increase of the growth energy cost. The threshold for effects on survival was estimated at 73.14 mg kg^{-1} , and the threshold for effects on energy budget (i.e., sublethal effects) at 19.21 mg kg^{-1} . This study demonstrates that DEB-TKTD models can be successfully applied to *E. crypticus* as a representative soil species, and may improve the ecological risk assessment for terrestrial ecosystems, and our mechanistic understanding of chemical effects on non-target species.

1. Introduction

For decades, ecotoxicological risk assessment (ERA) has used descriptive approaches based on dose-response curves at a single time point to assess the impact of chemicals on non-target organisms. The summary statistics resulting from descriptive methods (such as the LCx/ECx and NOEC) potentially have limited usefulness for science and risk assessment (Jager, 2011), mainly because they do not account for the time dimension, while toxicity is a process over time (Baas et al., 2010). This time dimension means that any calculated effects value derived

from those descriptive methods will vary in time. Clearly this is an unsatisfactory situation, because ecological risk assessment requires assessing effects of chemicals over time. Another shortcoming of the summary statistics traditionally used in ecological risk assessment is that they do not capture the physiological mode of action. The physiological mode of action (pMoA), however, has important consequences when extrapolating toxicant effects to the population level (Jager et al., 2014a, 2014b; Martin et al., 2014). For example, hypothetical stressors with different pMoAs that had equal effects on reproduction (i.e., assimilation, maintenance or reproduction) had effects ranging from a negligible

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decline in biomass to population extinction at the population level (Martin et al., 2014).

An alternative to summary statistics derived from classical toxicity tests is the use of mechanistic or process-based models which can provide better insight into the toxic effects on organisms over time, at all levels of biological organisation (Ashauer and Escher, 2010; Grimm and Martin, 2013; Jager et al., 2014a, 2014b; Vlaeminck et al., 2020). At the individual level, mechanistic effect models take the form of toxicokinetic–toxicodynamic (TKTD) models. Such approaches are more powerful than dose response models because they include the chemical concentration as well as the temporal dimension (Ashauer and Escher, 2010). They simulate the time course of processes leading to toxicity. For effects on survival, the leading framework is the general unified threshold model for survival (GUTS, Jager and Ashauer, 2018; Jager et al., 2011). For sub-lethal effects, the only TKTD models which have been used in ecotoxicology are based on dynamic energy budget (DEB) theory (Jusup et al., 2017; Kooijman, 2010). In 2018, the European Food Safety Authority (EFSA) accepted the GUTS framework as ready for applicability in ERA for aquatic organisms, and recognised the great potential of the DEBtox (i.e., DEB-TKTD) modelling approach. However, EFSA also concluded that the DEB-TKTD modelling approach was not yet ready to be used in regulatory risk assessment due to a lack of published examples of DEB-TKTD models for pesticides (EFSA, 2018). DEB-TKTD models have been successfully applied on several aquatic organisms including fish (Beaudouin et al., 2015; David et al., 2019) and invertebrates (Billoir et al., 2011; Muller et al., 2010). However, for soil organisms, application cases are much rarer, and only a very few examples are available in the literature for earthworms (Bart et al., 2019, 2020; Jager and Klok, 2010), springtails (Jager et al., 2004), and nematodes (but usually exposed in aqueous media for practical reasons) (Jager et al., 2005; Margerit et al., 2016; Schultz et al., 2021).

Enchytraeids (Annelida: Oligochaeta) are soil organisms, commonly found in a wide range of soils across the world. They belong to the soil mesofauna, having a body diameter between 0.1 and 2 mm (Gobat et al., 2004). Enchytraeids play key ecological roles in soils, contributing to litter fragmentation and organic matter decomposition (Marinissen and Didden, 1997; Bart et al., 2018; Pelosi et al., 2021a, 2021b). Moreover, they are involved in soil formation processes by increasing soil porosity and enhancing soil aggregation by mixing mineral and organic particles (Topoliantz et al., 2000). For these reasons, they have been recognised as indicators of soil quality and soil management impacts (Pelosi and Römbke, 2016). Despite their ecological importance and recommendations for their use in standardised toxicity tests (ISO 7, 1638, 2014; OECD 220, 2016), the toxicity of pesticides towards enchytraeids remains poorly documented mainly due to technical issues to experiment in soil with such small organisms (Römbke et al., 2017). To date, no DEB-TKTD modelling approach has been tested on these key soil organisms. While the data acquisition to calibrate a DEB-TKTD model on earthworms takes months of experiments (e.g., Anderson et al., 2013) due to their long-life cycles in contrast the life cycles of enchytraeids are much shorter and thus calibration data can be rapidly generated. For example, the generation time is 4 weeks for the model species *Enchytraeus crypticus*. The development of a DEB-TKTD modelling approach with this species could, therefore, facilitate efficient ecological risk assessment for terrestrial annelids.

In this investigation we developed an experimental design capable of providing the data required to calibrate a DEB-TKTD model for *E. crypticus*. A toxicity test following this design was then performed with the pyrethroid insecticide cypermethrin. The data from this study were subsequently used to calibrate the most-simplified DEB-TKTD model (DEBtox, Jager, 2020). Finally, we show how the calibrated model can be used to calculate standard ecotoxicological metrics such as the EC₅₀, and also to derive mechanistically important information, such as the physiological mode of action (pMOA) for the chemical.

2. Material and methods

2.1. Test organism, chemicals and experimental design

We used *Enchytraeus crypticus* (Enchytraeidae; Oligochaeta; Annelida), a model species in ecotoxicology (OECD 220, 2016). Individuals were originally sourced from the laboratory of the Department of Ecological Science, Vrije Universiteit, Amsterdam, The Netherlands, and were maintained in culture at the UK Centre for Ecology and Hydrology, Wallingford (UK). See [supplementary material](#) for more details.

2.2. Experimental procedure

The experimental design to collect data on the effects of cypermethrin on the *E. crypticus* cycle over time was inspired by the protocol described in [Bicho et al. \(2015\)](#).

2.2.1. Test soil, chemical and spiking

The standard natural soil LUFA 2.2 (Speyer, Germany) was used. Main characteristics of the soil can be summarised as follows: pH (CaCl₂) of 5.5, 44.5% of maximum water-holding capacity (WHC max), 4% organic matter content, and a grain size distribution of 6% clay, 14% silt and 80% sand.

We selected the insecticide cypermethrin (CAS; 52315–07–8, purity; 98%, obtained from Sigma Aldrich (St. Louis, MO, US)), a synthetic pyrethroid insecticide used in large-scale agricultural applications and in consumer pest control products. We selected this pesticide because there was data available in the literature regarding its toxicity toward *E. crypticus* in soil with an LC₅₀ (14 days) value of 31.4 mg kg⁻¹, and an EC₅₀ (28 days) for reproduction of 32.6 mg kg⁻¹ (Hartnik et al., 2008; Testa et al., 2020).

The soils were spiked with a stock solution of cypermethrin dissolved in acetone, so that 1 mL of acetone carrier would result in the desired final concentration. The spiked soil was left to evaporate until dry, approximately 30 min. The control treatment was spiked in the same manner, using acetone without chemical added, to allow an assessment to ensure that the acetone did not influence the enchytraeid responses. Previously to this experiment, preliminary assays conducted using a negative control without solvent, i.e., just water, showed that the solvent had no effect on enchytraeids. Soils were then mixed to ensure uniform chemical distribution, and water was added to reach 50% of the water holding capacity. Test vessels (4 cm diameter, 3 cm high) were filled with 10 g (dry equivalent) of soil, and left to stabilise for one day. Eleven vessels were prepared for each treatment and sacrificed over time for data acquisition. In addition, for each treatment, three vessels were prepared in the exact same condition than the experimental samples, and were frozen (–20 °C) at day 0, 7, and 28. These samples were then defrosted, 0.5 g of soil sample were spiked with recovery standard tau-Fluvalinate for the quantification of cypermethrin using liquid chromatography coupled to a triple quadrupole mass spectrometer (Waters ACQUITY UPLC system coupled with a Waters XEVO TQ-XS Tandem Mass Spectrometer; UK) interfaced with a Unispray source in positive MRM mode and operated using Masslynx software (version 4.2). The measured cypermethrin concentration was used to model the true exposure of enchytraeids and used as input in the DEB-TKTD model. See [Supplementary material](#) for more detail of the chemistry analyses and the cypermethrin dissipation modelling.

2.2.2. Enchytraeids synchronisation and exposure

The cultures of enchytraeids were synchronised by transferring adults with a well-developed clitellum into fresh agar plates to lay cocoons for 2 days. We then collected hundreds of produced cocoons and incubated them for 9 days in artificial fresh water in 48 well plates with 5–10 cocoons per well. At day 0 of the experiment, hatched juveniles were moved to a new 48 well plate and counted to get 20 juveniles per well within 0.5 mL of artificial fresh water. This was achieved by

pipetting the hatchlings with a standard micropipette. The 0.5 mL of artificial fresh water containing 20 juveniles was next added onto the soil surface of each test vessel. The additional water increased the water holding capacity from 50% to 60%, which was then checked every week by weighing the vessels. The enchytraeids were fed weekly with 20 mg of oatmeal (crushed and sieved using a 100 μM mesh) per vessel, gently mixed into the soil. This amount corresponds to ad libitum feeding based on preliminary assays. All test vessels were stored in a climate room at constant temperature (20 ± 1 °C) in constant dark.

To collect data on *E. crypticus* growth and reproduction over time, the eleven prepared vessels were sacrificed at day 2, 5, 7, 9, 12, 14, 16, 19, 21, 23 and 28 (Fig. 1). These time points, selected on the basis of preliminary assays were the best compromise between the experimental effort and obtaining sufficient data to calibrate the model, even if the time points were not exactly distributed in time. At each sampling time point, the sacrificed vessels were filled with ethanol (98% v/v) to fix individuals, and 200 μl of Bengal rose were added to colour the enchytraeids. The ethanol-flooded soil was gently mixed and stored in the fridge at 4 °C to be analysed later. The vessels were wet sieved through stacked 250 μm and 100 μm mesh sieves. Any young juveniles were mainly retained on the 100 μm mesh sieve, while bigger juveniles, adults, and cocoons were retained on the 250 μm mesh sieve. For the late time points (day 23 and 28), in the control and in treatments without effect or low effects (i.e., 1, 4 and 16 mg kg^{-1}), we had second generation juveniles present (i.e., the first-generation adults produced cocoons which hatched within the test vessels). We did not include these reproduction data in the model because we cannot mix cocoons and juveniles into one accurate reproduction metric, because we cannot know what happened to hatchlings (e.g., random death may occur), and we here focused on one generation only. All individuals and cocoons extracted were placed in a petri dish filled with ethanol, and pictures were taken on a graph paper (e.g., Fig. S1, S2). Image J software was used to measure the length of adult and juvenile enchytraeids and the diameter of cocoons (Schneider et al., 2012). For practical reasons, we directly used this physical length for the DEB modelling, rather than the estimated volumetric length. In theory, as long as it is used consistently, and the animal does not change shape during growth, any length measure can be used for DEB-TKTD model parametrisation (Jager, 2020). An *E. crypticus* cocoon may contain a variable number of eggs, ranging from few (2–5) to more than twenty (personal observation). Therefore, to accurately estimate the reproduction over time, we chose to estimate the number of eggs to characterise reproduction, rather than using the absolute number of cocoons. For this purpose, we estimated the volume of the cocoons based on their diameter (as an average of two measurements), considering them as spheres. Then we divided the cocoon volume by the estimated volume of a hatchling *Enchytraeus crypticus*

(estimated based on measured values for 20 individuals), which was an average of 0.0075 mm^3 , and used this value to estimate the number of eggs in each cocoon.

2.3. DEB-TKTD model

The model used is the most-simplified DEB-TKTD model presented in Jager (2020). For more clarity we present a summary of the model. In the model, food is taken up by enchytraeids from the soil, and a fraction κ of the assimilated energy is used for the soma (growth and somatic maintenance), whereas the remainder ($1 - \kappa$) is used for maturation (of juveniles), maturity maintenance, and reproduction (in adults). The growth equation is written as follows:

$$\frac{dL}{dt} = r_B(fL_m - L) \text{ with } L(0) = L_0 \quad (1)$$

Where L (mm) is the body length, r_B (d^{-1}) is the von Bertalanffy growth constant, $f(-)$ is the scaled functional response, L_m (mm) is the maximum body length and L_0 is the body length at the start of the experiment. Because enchytraeids present a slow initial growth phase, f is not a fixed value but is calculated with a hyperbolic relationship with body length to account for a food limitation for juveniles (please see the discussion in the results section, below).

$$f = \frac{1}{1 + \frac{L_f^3}{L^3}} \text{ Eqn} \quad (2)$$

Where L_f (mm) is the body length at half-saturation feeding.

For reproduction the compact model equation is written as follows:

$$R(L \geq L_p) = \max(0, R_m \frac{fL_m L^2 - L_p^3}{L_m^3 - L_p^3}) \quad (3)$$

$$R(L < L_p) = 0 \quad (4)$$

$$\frac{dR_c}{dt} = R \text{ with } R_c(0) = 0 \quad (5)$$

Where L_p (mm) is the physical length at puberty, R_m (number of eggs d^{-1}) is the maximum reproduction rate, and R_c is the cumulative reproduction (total number of eggs).

A mortality background h_b (d^{-1}) is also added to account for mortality not related to the toxic compound.

Together, r_B , f , h_b , L_f , L_m , L_p , and R_m form the physiological parameters of the DEB-TKTD model.

A TK-TD model accounts for the accrual of, and recovery from, damage (toxicodynamics, TD), related to the uptake, distribution, biotransformation, and elimination of the chemicals in the organisms (toxicokinetics, TK). We do not have information on body residues (measurements or predictions); therefore, the TK and TD part are combined into a one compartment model linking the external concentration to the damage, over time. Written as follow:

$$\frac{dD_s}{dt} = k_d(C_s - D_s) \quad (6)$$

Where D_s (mg kg^{-1}) is the damage level (scaled by the external concentration in soil), C_s (mg kg^{-1}) is the total concentration in soil (cypermethrin dissipation was modelled with a first-order kinetic model fitted to the chemistry data, see supplementary material), and k_d (d^{-1}) is the dominant rate constant describing the dynamics of the “scaled” damage, and represents the one-compartment approximation of the “true” two-compartment behaviour (TK and damage dynamics).

From the scaled damage level, a dimensionless stress level is calculated (Eq. 7), and modifies the value of one or more of the DEB-TKTD physiological parameters (Jager, 2020). The DEB-TKTD compact model with compound parameters offers access to four metabolic

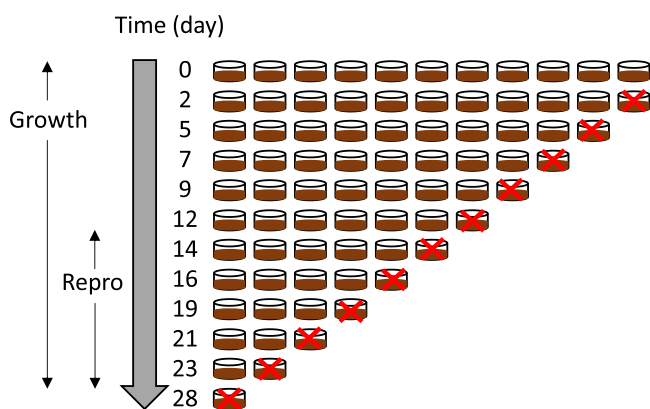


Fig. 1. Destructive sampling approach for the monitoring of *Enchytraeus crypticus* survival growth and reproduction in soil over time. Red cross means a sample is sacrificed for each treatment.

processes to be affected by the chemical: assimilation, maintenance, growth and reproduction (see [supplementary material](#) for more details). The affected metabolic process is generally referred to as a physiological mode of action (pMoA).

$$s = b_b \max(0, D_s - z_b) \quad (7)$$

Where s (-) is the stress level, b_b (kg mg^{-1}) is the effect strength on the energy budget, and z_b (mg kg^{-1}) is the damage threshold for effects on the energy budget.

We assume the same damage type affects both sublethal and lethal toxicity. To model survival a module from the GUTS framework is added: the stochastic death version:

$$h = b_s \max(0, D_s - z_s) \quad (8)$$

$$\frac{dS}{dt} = -(h + h_b)S \text{ with } S(0) = 1 \quad (9)$$

Where S (-) is the survival probability over time, h (d^{-1}) is the hazard rate, b_s ($\text{kg mg}^{-1} \text{d}^{-1}$) is the effect strength on survival (also known as killing rate, b_w , in the GUTS framework), and z_s (mg kg^{-1}) is the damage threshold for survival.

Together, k_d , b_b , z_b , b_s and z_s form the five toxicological parameters of the DEB-TKTD model.

2.4. Model fitting

All model calculations were performed in Matlab 2021 with the BYOM v.6.0 modelling platform (<http://www.debtox.info/byom.html>), with the package DEBtox2019. The optimisation of the parameter values was performed with the parameter-space explorer (Jager, 2021). This algorithm combines grid search, a genetic algorithm, and likelihood profiling, giving the Confidence intervals (CIs) of the parameter values. Regarding exposure, cypermethrin dissipates over time, therefore individuals are exposed to a time-varying concentration that is modelled following a first-order kinetic as described in the [supplementary material](#) (Fig. S1). For the survival data, each time point is a different population, therefore survival may increase in time in independent observations which does not make ecological sense; to address this, we used an independent binomial likelihood function, recently implemented in the BYOM platform. To produce CIs on the model curve, a sample from the parameter space explorer is used. We first fitted the physiological parameters of the model to the control condition. Next, we fitted the toxicological parameters to the entire data-set (i.e., on survival, growth and reproduction data together), keeping the physiological parameters fixed to their best fitted value on the control treatment. This procedure ensures that the model does not compensate for a poor representation of the treatment data by changing control behaviour.

Finally, the best pMoA (or combination) was determined according to the goodness of fits, with the Minus Log-Likelihood (MLL), and the Akaike Information Criterion (AIC).

3. Results and discussion

3.1. Modelling *Enchytraeus crypticus* life cycle

The OECD 220 guideline for testing chemicals on enchytraeid survival and reproduction (OECD 220, 2016) sets out the following validity criteria for performance in the controls: firstly, adult mortality should not exceed 20% at the end of the first three weeks of the test; secondly, for reproduction, an average of 2.5 juveniles should be produced per adult. In our experiment, the background mortality in the control treatment was an average of 5.4%, and never exceeded 15% (i.e., 17 individuals retrieved among the 20 added at day 0) even at the maximum 28 d of exposure (Fig. 2). Reproduction (i.e., the estimated egg production) in the controls was 5.3, 19.1, 33.2 and 49.4 eggs/adult at day 14, 16, 19 and 21 respectively. Although this test was not carried out following every aspect of the OECD 220 guideline, fulfilment of the control validity criteria set out there indicates that the test system we used does not detrimentally impact adult survival or reproduction, and therefore is appropriate for the destructive sampling approach we propose.

In DEB models, maintenance requirements take precedence over growth. Because food uptake is assumed to be proportional to the surface area, whereas maintenance is proportional to volume, a von Bertalanffy curve with a maximal asymptotic length is expected (under constant conditions). The experimental results showed a maximum asymptotic length, estimated at 9 mm (Table 1), but a slow initial growth phase in *Enchytraeus crypticus*, leading to a sigmoidal curve. We assumed a food limitation at an early stage, and the addition of a size-dependent food limitation for juveniles provided an accurate simulation of the growth (Fig. 2). It is worth noting that such a growth curve might also be explained by metabolic acceleration phenomena, as mainly observed in insects (Kooijman, 2014). However, such slow initial growth curves have been previously observed in other worms and was explained by food limitation. For instance, in nematodes, detailed physiological studies showed a food limitation imposed by the size of the buccal cavity (Avery and Shtonda, 2003). Enchytraeids belong to the subclass Oligochaeta in the phylum Annelida, like earthworms, and for which this initially slow growth was also observed (Bart et al., 2019; Boström and Lofs-Holmin, 1986; Fernandez et al., 2010). This restriction was explained by the size of food particles they can reach and ingest in the early juvenile period (Bart et al., 2019). However, despite the fact the food limitation works well to simulate enchytraeids growth, more dedicated research is required to confirm that food limitation is responsible for the initial slow growth.

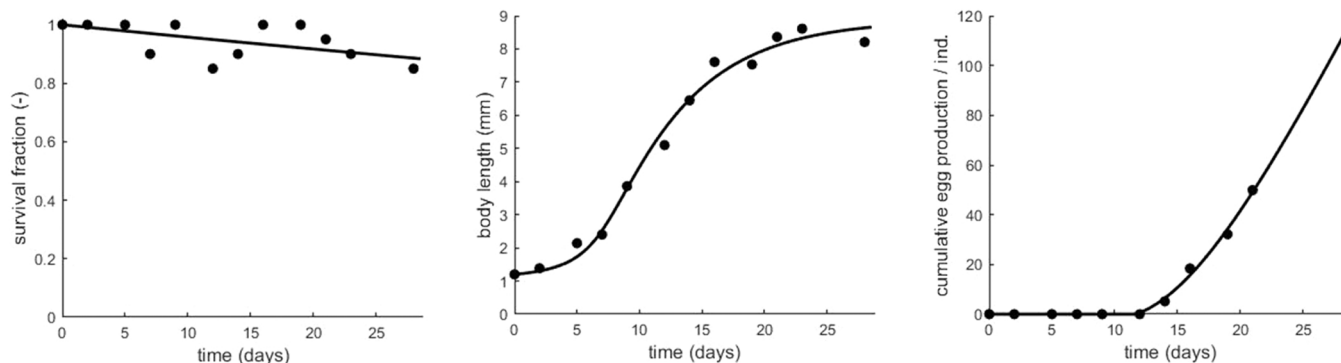


Fig. 2. Background mortality (left plot), growth (middle plot) and cumulative egg production (right plot) over time for *Enchytraeus crypticus* in an artificial LUF4 2.2 soil at 60% water holding capacity, incubated at 20 ± 1 °C and fed ad libitum with crushed oat meal. Points are the data (mean of 20 individuals in each destructively sampled test vessel) and the lines are the DEB model.

Table 1

Parameter values, with the confidence intervals, of the DEB-TKTD model. Physiological mode of action: increase of the growth energy cost.

Symbol	Description	Value (CI)	Unit
<i>DEB-TKTD physiological parameters (fitted to the control treatment)</i>			
L_0	Initial body length	1.2 (fixed)	mm
L_p	Body length at puberty	5.4 (4.9–5.7)	mm
L_m	Maximum body length	9 (8.6–9.8)	mm
r_B	Von Bertalanffy growth constant	0.17 (0.12–0.20)	1/d
R_m	Maximum reproduction rate	10.5 (9.2–12.8)	eggs/d
L_f	body length at half-saturation feeding	2.1 (2.0–2.2)	mm
h_b	Background hazard rate	0.4 (0.3–0.7) 10^{-2}	1/d
<i>Toxicological parameters</i>			
k_d	Dominant rate constant	10* (1.558–10)*	1/d
z_b	Threshold energy budget	19.21 (18.36–19.90)	mg/kg
b_b	Effect strength energy-budget	0.036 (0.032–0.040)	kg/mg
z_s	Threshold survival	73.14 (65.07–78.49)	mg/kg
b_s	Effect strength survival	9.3 (7.3–11.6) 10^{-4}	kg/mg/d

* Boundary of the parameter space explorer

Regarding the reproduction, the model captured the cumulative egg production well (Fig. 2). *E. crypticus* continuously produced cocoons once they reached their pubertal size, estimated at 5.4 mm length in our study (Table 1), after 12 days.

3.2. Cypermethrin effects modelled with the DEB-TKTD model

Cypermethrin had an effect on survival with a threshold for survival estimated at 73.14 mg kg⁻¹, and effects can be seen in Fig. 3 for the two highest tested concentrations. Regarding sub-lethal effects, the model fitting of all potential pMoAs clearly showed that the most likely pMoA

for cypermethrin on *E. crypticus* is an increase of the growth energy cost (Table S1, MLL = 1130.2, r2 growth = 0.9729, r2 reproduction = 0.9701, r2 survival = 0.9212). To our knowledge, this is the first-time only growth costs have been reported for a pesticide. Effects on growth are generally found together with an effect on reproduction costs (Alda Alvarez et al., 2006; Jager and Selck, 2011). Cases where only growth costs have been reported as the pMoA were for the metal cadmium and copper (Ashauer and Jager, 2018). However, it is important to note that the number of applied DEB-TKTD cases is still very limited, and it is likely this pMoA will be highlighted for other chemicals in the future. A hypothesis for the effects of cypermethrin on growth due to an increase of the growth energy cost in *E. crypticus* revealed by the DEB-TKTD model is not fully developed within known information on the effects of this chemical. At the molecular level, cypermethrin is known to act on the central nervous system, where it prolongs the opening of sodium channels leading to hyper-excitation of the central nervous system (Singh et al., 2012). Furthermore, this insecticide also modulates chloride, voltage-gated calcium and potassium channels. These interactions can alter the activity of glutamate and acetylcholine receptors and adenosine triphosphatases, inducing DNA damage and oxidative stress in the neuronal cells (Singh et al., 2012). Moreover, studies have shown cypermethrin modulates levels of neurotransmitters, including gamma-aminobutyric acid (Manna et al., 2005) and dopamine (Nasuti et al., 2007). The mechanisms of how these effects at a molecular level can lead to impacts on growth at the level of the organism are not yet established. However, a plausible hypothesis that may explain the pMoA of cypermethrin is related to the cell cycle. Huang et al. (2016) found that cypermethrin caused cell cycle arrest and apoptosis, and these effects were also reported by Ji et al. (2021). The energy invested in the creation of new cells would be higher if a fraction of the cell cycles is not completed due to an effect from cypermethrin.

The effect on growth due to cypermethrin exposure, has been previously reported in various other species such as fly larvae (*Hermetia*

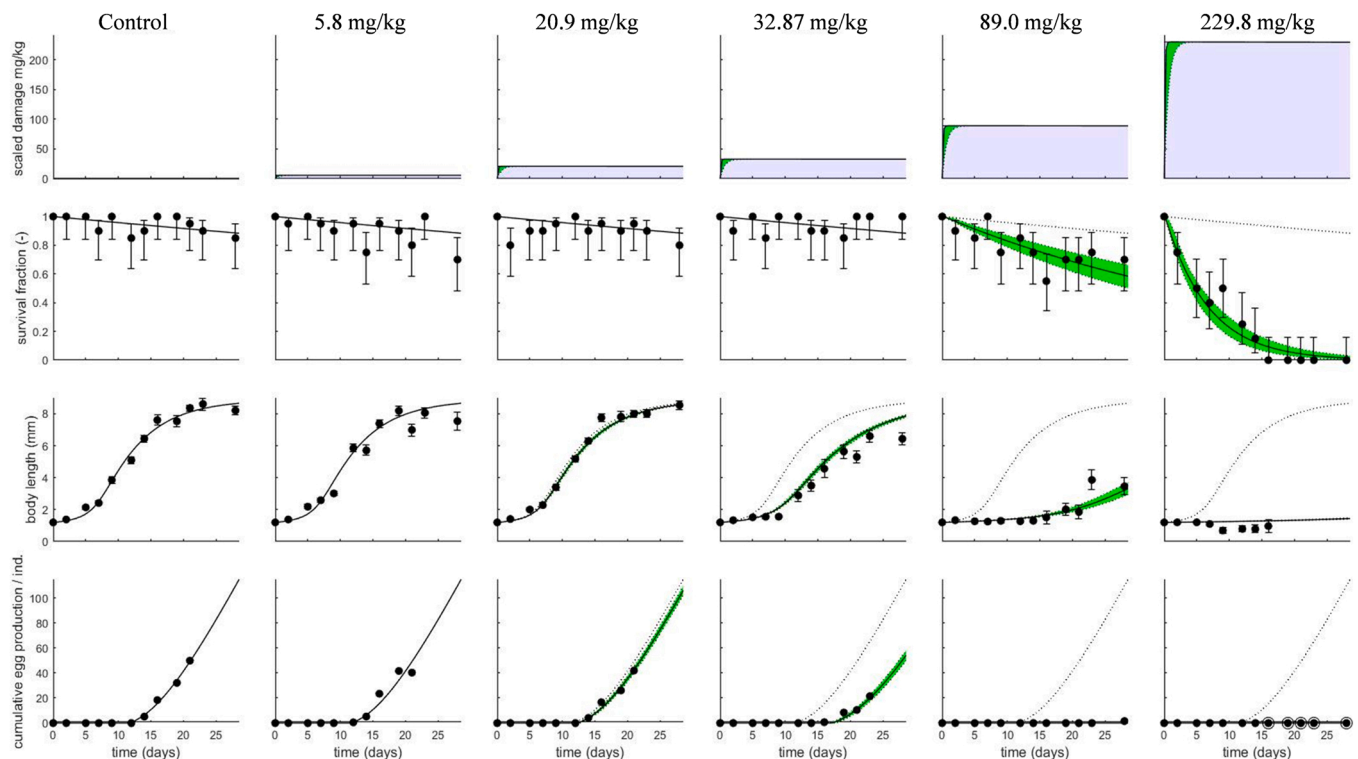


Fig. 3. Simulations with the DEB-TKTD model (solid lines, confidence intervals as green areas) with the physiological mode of action: G (increase of the growth energy costs) for *Enchytraeus crypticus* exposed to cypermethrin in a LUFA 2.2 soil. Shown are the scaled damage (first row), survival (second row), growth (third row), and reproduction (fourth row) over time. The points represent the measured data from the study (means \pm SE). The dashed line represents the modelled control treatment (no cypermethrin).

illucens, Meijer et al., 2021), *Daphnia magna* (Gottardi et al., 2017), copepods (Zhou et al., 2019), and the freshwater fish *Oreochromis niloticus* (Majumder and Kaviraj, 2017). However, effects on growth can also be attributed to the pMoAs decrease in assimilation and/or increase in maintenance cost in a DEB-TKTD context. In our case study, the pMoA increase in growth costs due to cypermethrin is, indirectly, not characterised only by effects on growth. Clearly, cypermethrin reduces the egg production as well (Fig. 3). But these effects on reproduction appear to be due to a delay in growth rate, which triggers a delay in reaching puberty and starting reproduction compared to the control treatment. When comparing animals in the control and the treatments at the same body length, the reproduction rate is quite similar (Fig. 3). The DEB-TKTD model allows us to mechanistically separate the direct and indirect effects of cypermethrin on enchytraeids' life history traits. It is important to note that an impact on growth will ultimately have an impact on population dynamics, due to the delay that is caused to reproduction.

The dose-response of and the threshold for toxic effects of cypermethrin to *E. crypticus* in soil reported here might be different in another soil type, because toxicity is highly dependent on soil characteristics which influence bioavailability (Bradham et al., 2006; Hennig et al., 2022). The threshold for effects on the energy budget under the conditions of our study was estimated at 19.21 (18.36–19.90) mg/kg (Table 1). This level is 400 times higher than concentrations reported in arable soils (maximum of 50.9 µg/kg in Pelosi et al., 2020), indicating that the risk from cypermethrin to enchytraeid populations is probably low under field conditions.

The growth data showed that the impact of cypermethrin on growth appears more pronounced for the younger juvenile enchytraeids than for bigger individuals (Fig. 3, in the treatment 16 mg kg⁻¹, individuals barely grow up to day 9, and then the curve is nearly similar to the control treatment). This result is linked to the DEB model structure, where food limitation will always generate stronger effects on growth (relative to the control) for certain pMoAs (assimilation, maintenance and growth costs) (Zimmer et al., 2012). This phenomenon interpreted with classical statistical analyses could be called a "synergistic" interaction between the toxicant effects and the food limitation, while the use of a DEB-TKTD model analysis in this case study identified a mechanistic explanation of this phenomenon.

Enchytraeids grew and reproduced throughout the exposure to cypermethrin. This continued production of new biomass and changes in dimension of the organism could affect the toxicokinetics and/or the scaled damage dynamics. In the context of DEBtox, several feedbacks on the one compartment TKTD model can be considered including: changes in surface:volume ratio of the organism; growth dilution (i.e., when an individual grows in volume, the chemical internal concentration decreases); and elimination of internal concentrations with reproduction. We tested the addition of these feedbacks on the scaled damage equation (Eq. 6), as presented in Jager (2020). Model fits were equally good when compared to model fits without feedbacks (considering the pMoA G, the best fit was found to occur with the feedback dilution by growth: MLL = 1128.84; whilst the MLL was 1129.02 without feedback). Similarly, these fits indicate that the simplest approach with no feedbacks is the most parsimonious approach, i.e., increasing the complexity does not lead to significant improvement in prediction. This result suggests that the scaled damage in the model reflects a damage process rather than a toxicokinetic process, since the latter should be affected by growth (Jager, 2020). Thus, the dominant rate constant ' k_d ' in Table 1 probably represents the damage repair rate rather than the toxicokinetic elimination rate.

3.3. Strengths, weaknesses, and recommendations for future improvement of the method for ecological risk assessment

This case study demonstrates that the simplest DEB-TKTD model can be applied not only to aquatic organisms, but also to soil organisms, and

the use of *E. crypticus* is promising due to its high ecological importance in contributing to soil functions (Pelosi et al., 2021a, 2021b; Pelosi and Römcke, 2016). The novel experimental design successfully provided the data required to calibrate a DEB-TKTD model, and allowed a complete TKTD analysis of the effects on survival, growth and reproduction, simultaneously over time. The destructive sampling technique provides independent observations (i.e., independent populations of test organisms are sampled) at each time point, making the analysis and model calibration more robust compared to other experimental designs, because the likelihood functions assume independence, which is normally violated in regular DEB-TKTD model calibration with aquatic organisms (in which the same individuals are monitored throughout their life cycle).

The experimental approach presented can be improved in the future. For instance, the length measurement with the ImageJ software could be readily automated (Duckworth et al., 2019). For reproduction, we monitored the overall cocoon production but did not take into account a potential hazard to embryonic development due to transfer of cypermethrin from the mother to the eggs, nor the production of unviable cocoons. Furthermore, the potential for accumulation of cypermethrin into cocoons through soil exposure was also not accounted for, which could lead to a decrease in hatching success. These effects could have an ecologically relevant impact, and could be addressed by investigating the exposure of healthy cocoons in contaminated soils and monitoring their hatching success, as presented in Bicho et al. (2015). To also measure the hazard to embryonic development, monitoring of juvenile production could be added to the experimental design (as is presented in the OECD guideline OECD 220, 2016). However, retrieving of hundreds of juveniles at several time points is more time consuming than retrieving cocoons (personal observation), and monitoring of both requires additional time, therefore there are resource implications for extending studies to monitor this parameter. Further research would be needed to find a balance between experimental effort and the minimum data required for the calibration of a DEB-TKTD, which provides a much better insight than ad hoc analyses with traditional summary statistics. Where needed (for example for comparative purposes) the model can be used to derive traditional ecotoxicological metrics (LC_x and EC_x for reproduction and growth) at any time point (Table 2). From the calibrated DEB-TKTD model we calculated an LC₅₀ (14 days) of 127 mg kg⁻¹ (CI 119–136, Table 2), which is in similar range than Hartnik et al. (2008) who found a LC₅₀ (14 days), estimated at 31.4 mg kg⁻¹. For reproduction, Testa et al. (2020) estimated an EC₅₀ (28 days) at 32.6 mg kg⁻¹, which is very close to our calculated EC₅₀ (28 days) of 31.5 mg kg⁻¹ (CI 30.6–32.5, Table 2). It is important to note that soil modulates toxicity through bioavailability, but can also affect organism performance independently of its role on contaminant bioavailability, for example due to food availability (Jegade et al., 2019). Therefore, it is likely that our calibrated model would not accurately predict effects of cypermethrin on enchytraeids life cycle in a soil with different properties. This specificity of soil ecotoxicology

Table 2

EC₁₀ and EC₅₀ for survival (= LC₁₀ and LC₅₀), growth and reproduction of *Enchytraeus crypticus* exposed to cypermethrin in a LUFA 2.2 soil for 28 days. Values are derived from a calibrated dynamic energy budget model coupled with a one compartment toxicokinetic-toxicodynamic model (DEB-TKTD).

Time (days)	EC10 (mg/kg)	EC50 (mg/kg)
Survival (LCx)		
14	81.4 (74.6–86.2)	127 (119–136)
28	77.2 (69.8–82.0)	100 (94.4–105)
Growth		
14	22.7 (22.1–23.2)	39.7 (38.2–41.5)
28	33.2 (32.1–34.2)	72.2 (67.6–77.2)
Reproduction		
14	19.6 (18.8–20.3)	21.5 (20.8–22.0)
28	21.4 (20.7–21.9)	31.5 (30.6–32.5)

applies not only to our approach, but to all soil ecotoxicological experiments, and also to dose response models.

The calculation of EC_x values with a dose-response model for chemicals that undergo substantial dissipation throughout the test period is currently problematic, because calculations are usually based on nominal concentrations (or measured initial concentrations), while organisms may be exposed to lower concentrations at the end of the test, due to chemical dissipation. Conversely, calculation of these metrics with the calibrated DEB-TKTD model is convenient and robust because

the EC_x calculation using the calibrated model forces the modelled exposure (i.e., the concentrations) to be constant. In addition, EC_x values can vary over time (Fig. 4); LC_{50} values strictly decrease in time because of the binary nature of the response, and because dead animal can't recover. For sub-lethal effects (i.e., EC_{50} growth and reproduction), it is possible for values to increase or decrease over time. More specifically, the EC_x for body length has a minimum due to the pMoA G: toxicant stress makes it more expensive for the organism to grow, but does not affect the final size, leading to a theoretically infinite EC_x for

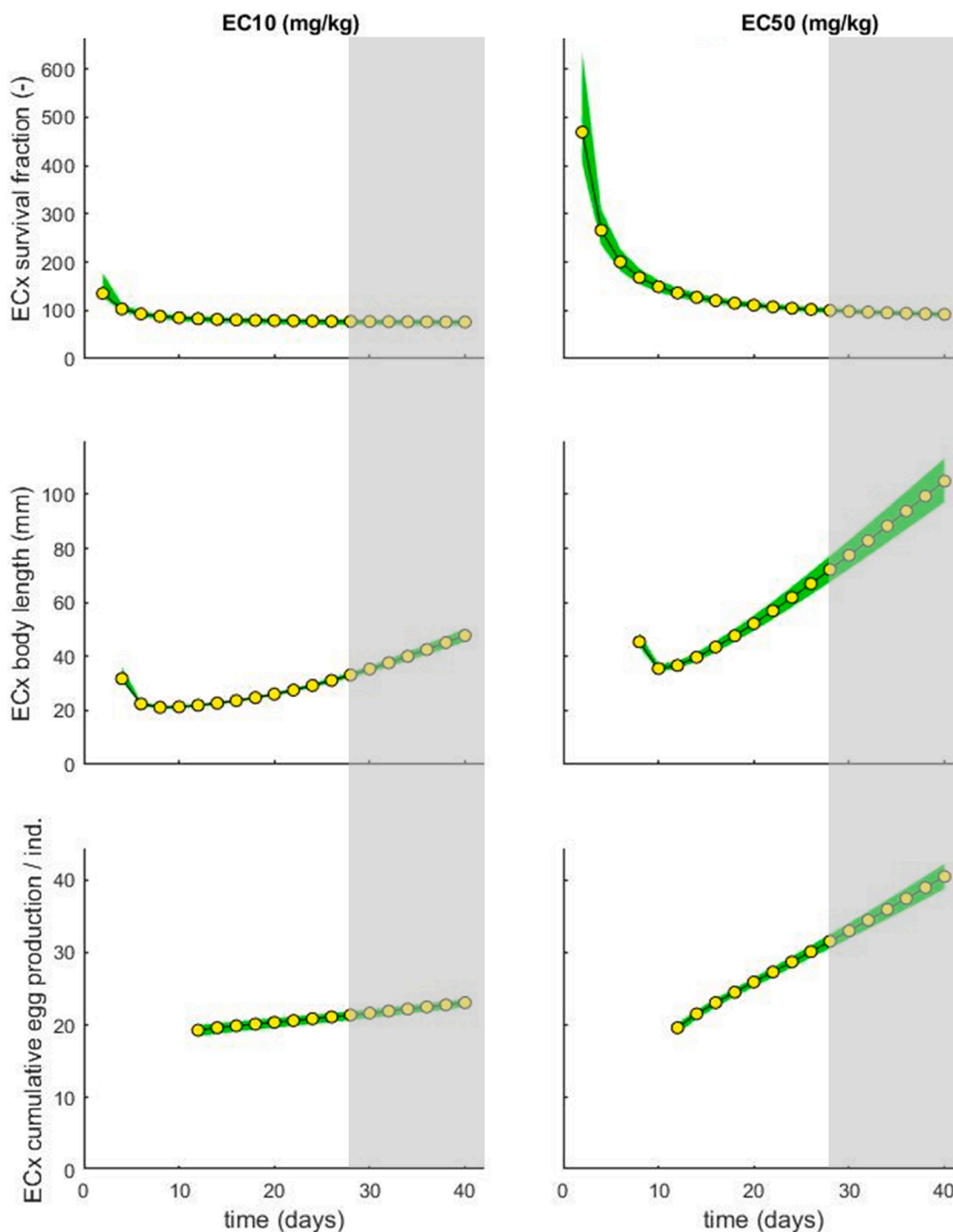


Fig. 4. Prediction of the EC_{10} and EC_{50} (mg/kg) for survival, growth and reproduction as a function of time, with 95% confidence interval. Predictions are from the DEB-TKTD model with the physiological mode of action: G (increase of growth energy costs) fitted to survival, growth and reproduction data of *Enchytraeus crypticus* exposed to cypermethrin in a LUFA 2.2 soil. The grey area represents the extrapolation in time of the EC_x , beyond the experimental timeframe.

body length if we wait long enough. The EC_x for reproduction increases over time, which is also related to the pMoA G, and the fact that cumulative reproduction over time is used in the DEB-TKTD models.

This study case demonstrates the strengths of the DEB-TKTD model, and it is of particular interest from an ERA perspective (Baas et al., 2018), because it provides a convenient way of quantitatively assessing effects on sublethal endpoints or life-history traits without the need to fully elaborate biological details nor information about the stressor. The calibration of the DEB-TKTD model, done here with cypermethrin, provides relevant metrics for risk assessment through the threshold concentrations for lethal and sublethal effects which are calculated as time-independent parameters. The calibrated model can be used to predict effects under untested scenarios such as time-variable exposures, or combined effects of different food levels and cypermethrin concentration.

Supplementary material

The [Supplementary material](#) contains the *Enchytraeus crypticus* culture condition, the equations regarding the different pMoAs, the method for the chemistry analyses as well as the modelling of cypermethrin dissipation in soil, the goodness of fit for the different pMoAs, examples of pictures for the length measurements with imageJ, the parameter space plot, an all-in-one plot with the different concentration tested, and the observed vs simulated data plot. It also contains a file with the raw data, and the MATLAB code to reproduce the analyses. The files are within BYOM V6, → DEBtox2019 → simple compound. See <http://www.debtox.info/byom.html> for more information.

CRedit authorship contribution statement

Sylvain Bart: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Tjalling Jager:** Methodology, Software, Resources, Writing – review & editing. **Stephen Short:** Resources, Project administration, Writing – review & editing. **Alex Robinson:** Investigation, Resources. **Darren Sleep:** Validation, Investigation. **M. Glória Pereira:** Validation, Investigation, Resources. **David J. Spurgeon:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Roman Ashauer:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Roman Ashauer reports a relationship with Syngenta Crop Protection AG that includes: employment.

Data availability

The data and modelling code are provided.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2023.114499](https://doi.org/10.1016/j.ecoenv.2023.114499).

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