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RESEARCH ARTICLE

Contributions of egg production and egg hatching to the total toxicity of teflubenzuron in *Yuukianura szeptyckii* (Collembola) in soil toxicity test

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Abstract

In the standard ISO soil toxicity test using Collembola, adult survival and juvenile production are the only endpoints that can be attainable. The information on egg production and egg hatching cannot be investigated in the ISO test. To overcome this limitation, in this study, the effects of teflubenzuron on life history parameters of *Yuukianura szeptyckii* (Collembola) were investigated with a compressed soil test. Teflubenzuron is an insect growth regulator and has a negative effect on egg production, and egg hatching process of arthropods. LC_{50} decreased with increases in exposure period from 6.97 mg/kg in the third week to 3.60 mg/kg in the fourth week. The EC_{50} for egg and juvenile production was 0.57 mg/kg and 0.26 mg/kg, respectively. The hatching rate decreased significantly from 46 to 7% as the concentration increased from 0.25 to 1.00 mg/kg, respectively, and the molting frequency was significantly affected only at > 4 mg/kg. The toxic contribution rate (TCR) was defined as the ratio of juvenile production at an exposure concentration compared with the control, and a simple life history model was developed for TCR estimations. At the lower concentrations (< 0.3 mg/kg), the hatching rate reduction was a main contributor to the total toxicity, but the adult mortality and egg production reduction were the main contributors at the higher concentrations (> 2.0 mg/kg). The contribution of egg production reduction remained relatively constant. Since collembolan populations in the soil can be composed of various developmental stages, the differences in the sensitivity to chemicals depending on the developmental stages should be included in the assessment of the toxic impact on soil ecosystems.

Keywords IGRs · ISO · Compressed soil test · Life history · Toxic contribution rate

Introduction

In soil ecotoxicology with collembolans, the reproduction output of collembolans is considered to be an effective and ecologically relevant parameter to assess chemical toxic effects in terrestrial ecosystems (Scott-Fordsmand et al. 1997; Son et al. 2007). ISO (1999) has proposed a standardized collembolan ecotoxicological laboratory test protocol, mainly focused on two endpoints of adult survival and juvenile reproduction. The test container should have a 30 g volume holding of fresh soil with a 2–4 cm soil depth, which allows collembolans to burrow into the soil, and complete their life cycles in the soil. The soils were destructively sampled to extract adults and juveniles using the water-flooding method after a 28-day exposure (Son et al. 2007). Although this protocol has an advantage of a simple procedure that requires little attention during the exposure period (Van Straalen and Van Gestel 1993), it has the limitation that the information on crucial biological processes of the collembolans, such as egg laying and egg hatching, are nearly impossible to observe during the exposure.

The juvenile production is considered to be a most comprehensive endpoint in the collembolan ecotoxicity test

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because the numbers of eggs laid per surviving adult, and their egg hatchability determine the juvenile production (Son et al. 2007). However, it is hard to determine whether these toxic effects on the juvenile production are mainly from the reduced egg production or reduced hatching rate. In the case of selective insecticides, such as insect growth regulators (IGRs), it includes a variety of chemical class with different mode of actions that interfere some way with processes of growth and development to reduce target population (Darvas and Polgar 1998; Graf 1993). Consequently, the information on egg production and their hatchability in collembolan toxicity test should be included as endpoints to achieve an ecologically relevant risk assessment of chemicals that have multiple effects.

Among the IGRs, teflubenzuron [1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)urea] has been used broadly to control a wide range of insect pest populations, including lepidopteran, dipteran, and coleopteran pests, on cotton, coffee, soybeans, and tomatoes in many countries since the late 1970s (Darvas and Polgar 1998; Medeiros et al. 2013). It is known to inhibit chitin synthesis, interfering with larval development by causing deformations in the cuticle layers (Cycoń et al. 2012). Although the mode of action of teflubenzuron has remained elusive, the insects exposed to teflubenzuron show a variety of symptoms that affect the epidermis, tracheal system, midgut epithelium, and oocytes (Merzendorfer 2013). Subsequently, this causes adverse effects on the major life history parameters, including the molting process, fecundity, and egg hatching success (Acheuk et al. 2012; Fisk and Wright 1992; Horowitz et al. 1992). Because the adverse effects of teflubenzuron can occur in all chitin-synthesizing organisms, application of teflubenzuron to control insect pests can cause severe adverse effects on non-target arthropods. For this reason, toxic effects on non-target aquatic arthropods have been of concern (Olsvik et al. 2017). However, few studies have investigated soil arthropods (Cresci et al. 2018).

Since teflubenzuron is known to affect egg production and hatchability of arthropods simultaneously (Acheuk et al. 2012; Fisk and Wright 1992; Horowitz et al. 1992), a new toxicity test method that can investigate toxic effects on egg production, and hatchability is essential for accurate evaluation of teflubenzuron toxicity. To overcome the limitation of the current ISO test method for collembolans (ISO 1999), a compressed soil test method has been proposed and evaluated for various chemicals (Campiche et al. 2006; Jensen et al. 2001; Lee et al. 2018). On the compressed soil, collembolans cannot burrow in the soil, thus eggs, juveniles, and adults can be detected on the soil surface. All of the previous compressed soil toxicity studies have been supplemented to determine the toxic effects that could not be investigated by the ISO standard method, such as egg production, egg hatching rate, and juvenile mortality (Campiche et al. 2006; Jensen et al. 2001; Lee et al. 2018). The compressed soil test has never been attempted for the comprehensive toxicity assessments.

In this study, *Yuukianura szeptyckii*, a collembolan species belong to the family Neanuridae was used as a model organism to evaluate the toxicity of teflubenzuron. This species is suitable for evaluating toxic effects of teflubenzuron on compressed soil because biological parameters, such as adult survival, egg production, egg hatching rate, juvenile production, and molting frequency, were fully investigated on compressed soil at various temperature conditions (Lee et al. 2016). In addition, because of its large egg and body size (1.4–1.7 mm in length), distinctive orange body color, and no jumping, this species can be easily observed on the compressed soil surface (Fig. 1S).

The objectives of this study were to evaluate the toxic effects of teflubenzuron on *Y. szeptyckii* with various endpoints, including adult survival, egg production, egg hatching rate, and molting frequency on a compressed soil. Further, we aimed to develop an evaluation model to estimate how each affected life-history parameter by teflubenzuron exposure contributed to the decrease in juvenile production at various teflubenzuron exposure concentrations. In addition, the performance and sensitivity of the compressed test method were compared with the ISO standard method with two endpoints, adult survival and juvenile reproduction.

Materials and methods

Test organism and culture

Yuukianura szeptyckii individuals were collected from Ansan City, Gyunggi Province, Korea, near a stream in 2006, and maintained in the laboratory (Lee et al. 2016). This species was cultured in a glass jar (90 mm diameter, 90 mm height) filled with 140 g of OECD artificial soil (10% finely ground sphagnum peat, 20% kaolinite clay, and 70% sand, and with the pH adjusted to 6.0 ± 0.5) (OECD 1984) as a substrate at 25 ± 0.5 °C with continuous darkness. The water-holding capacity (WHC) of the soil was adjusted to 60%, and the soil pH was adjusted to 6.5 ± 0.5 with the addition of $CaCO_3$. Distilled water was added periodically, and jars were aerated weekly to maintain the fresh condition of the substrate. Granulated dry yeast was added to the cultures twice a week as food in small amounts to avoid spoilage due to excessive fungal growth.

Experimental chemical and solvent

Analytical standard of teflubenzuron (purity > 98.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). As its water solubility was low, a stock solution was prepared by dissolving teflubenzuron in acetone obtained from Sigma-Aldrich.

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Preparation and contamination of the soil substrate

Toxicity testing was conducted with artificial OECD soil (ISO 1999). WHC and pH in the soil were adjusted to 60% and 6.5 ± 0.5, respectively (OECD 2008). The quantity of the stock solution required to obtain the desired concentrations was added to a volume of acetone equivalent to the total mass of quartz sand (Campiche et al. 2006). The prepared acetone was added to the soil substrate and mixed thoroughly. The soil mixtures with the desired concentrations of teflubenzuron were kept under a fume hood for 12 h to completely evaporate residual acetone. All soils were prepared 1 day in advance and stored at room temperature.

Collembola toxicity test

Cohorts of *Y. szeptyckii* were obtained using a compressed OECD artificial soil substrate (Lee et al. 2016). Approximately 2 g of soil was placed in a Petri dish (60 mm diameter; 15 mm height) and compressed firmly with an acrylic disk (60 mm in diameter) to a depth of 2 mm. Fifty *Y. szeptyckii* adults were introduced on the surface of the compressed soil and placed at 25 ± 0.5 °C in continuous darkness. After 1 week, the eggs were transferred to a fresh soil substrate using a fine hair brush. The eggs were hatched after 10 days, and the juveniles developed to adults 6 weeks later. The resulting adults (6-week old) were used for all the toxicity tests.

A 28-day compressed soil test was performed to determine the toxicity of teflubenzuron to *Y. szeptyckii* at various endpoints. Further, the 28-day Collembola reproduction test in accordance with the ISO standard 11267 method was performed to compare the performance and sensitivity of the 28-day compressed soil test at two endpoints: adult survival and juvenile reproduction. A basic difference between these two test methods was the exposure pathway of teflubenzuron to *Y. szeptyckii*. In the ISO standard method, the entire body of *Y. szeptyckii* is exposed to teflubenzuron in the contaminated soil, but the compressed soil test only exposes chemicals to the soil surface through contact. On the compressed soil, the soil pore space was smaller than the body size of adult and juvenile *Y. szeptyckii*, preventing collembolans from burrowing (Lee et al. 2016). Thus, all life stages of *Y. szeptyckii* (egg, juvenile, and adult), including exuviae, can be detected on the soil surface. This procedure enabled recording of the numbers of eggs, hatched juveniles, and moltings per adult.

For the compressed soil test, 2 g (wet weight) of teflubenzuron spiked soil, with concentrations of 0 (control), 0.25, 0.5, 1, 2, 4, 8, and 16 mg/kg were placed in a Petri dish (60 mm diameter, 15 mm height, with covers) and compressed firmly to a depth of 2 mm. The WHC of soil in the dish was adjusted to 60% by adding distilled water as necessary.

Briefly, 140 compressed dishes (7 concentrations × 5 replicates × 4 weeks) were prepared at once and stored at 25 ± 0.5 °C in continuous darkness. At day 0, 35 dishes (5 dishes per concentration) were randomly selected from the stored dishes. Ten (6-week old) adults were introduced per dish and maintained at 25 ± 0.5 °C for 1 week. The number of eggs and exuviae in each dish was counted, and the exuviae were removed. Surviving adults from the original dish were transferred to a fresh dish containing the same concentration with 10 mg of dry yeast at the center of the dish. The original dish containing the eggs was kept under the same experimental conditions until all eggs had hatched (hatching took no longer than 28 days), and the resulting juveniles were counted. This procedure was continued for 28 days at weekly intervals. This procedure enabled accurate determination of the egg production, egg hatching rate, and adult-molting frequencies. Developmental periods of eggs and juveniles in *Y. szeptyckii* at 25 °C were 11.6 days and 22.5 days, respectively (Lee et al. 2016). Thus, if the dishes were censored 28 days after exposure, some portion of eggs laid during the first week could develop into adults. Further, inviable eggs laid during the early weeks (1–2 weeks) could decompose during the 28-day period. Moreover, degradation effects of teflubenzuron on reproduction and egg hatching rate of *Y. szeptyckii* could be evaluated during the test periods. Weekly toxic effects on the adult survival (LC_{50}), egg production ($EC_{50\ =\ egg\ production}$), juvenile production ($EC_{50\ =\ juvenile\ production}$), egg hatching rate ($EC_{50\ =\ egg\ hatching}$), and molting frequency (molts/individual/day) could be determined.

To evaluate the sensitivity and performance of the compressed soil test for teflubenzuron, the standardized Collembola reproduction test ISO 11267 (ISO 1999) was also performed. Each test container with a plastic lid (70 mm diameter, 75 mm height) was filled with 30 g (wet weight) of OECD artificial soil (pH = 6.5 ± 0.5 and WHC = 60%) without compressing the substrate. Ten adults were introduced into each container. Approximately 10 mg of dry yeast was added as the food at the beginning of the test and two weeks later. After 28 days, the test containers were flooded with distilled water and gently stirred with a spatula to allow drift of collembolans to the water surface. The number of adult and juveniles was counted under a stereo microscope. The experimental concentrations for this method were 0 (control), 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/kg. Five replicates per concentration were performed in this experiment.

For both toxicity test methods, two controls with acetone only and distilled water only were run in parallel to investigate the effect of acetone, a carrier solvent of teflubenzuron, on the biological performance of *Y. szeptyckii*.

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Determination of the teflubenzuron concentration in soil

To determine the weekly changes of teflubenzuron concentration in the soil during the 28-day exposure period, 16 mg/kg teflubenzuron in the soil, which is the highest nominal concentration tested in this experiment, was prepared. Soil samples were weighed to 30 g and Soxhlet-extracted for 12 h using 200 mL of *n*-hexane. The *n*-hexane extract was concentrated using a rotary evaporator and transferred to a vial. The solvent was completely evaporated under a gentle nitrogen stream, and the dried residue was re-dissolved in 3 mL of acetonitrile. Aliquot of 700 μL of acetonitrile solution was filtered using a syringe filter (polyetrafluoroethylene, 0.45 μm pore size) before the high-performance liquid chromatography (HPLC) analysis. The HPLC system was comprised of a quaternary gradient pump (Waters 600E, Milford, MA, USA), reverse phase C18 column (150 × 4.6 mm, 5 μm inner diameter, Fortis, Neston, UK), and a photodiode array detector (Waters 2998). The total flow rate was 1 mL/min (acetonitrile: water = 9:1, isocratic), and the wavelength of the detector was 254 nm.

Data analysis

Unless stated otherwise, all statistical analyses were conducted using SAS software, version 9.3 (SAS Institute 2011). In the compressed soil test, changes in egg numbers, hatching rates, and molting frequencies between experimental weeks were compared using two-way ANOVA with the week and concentration as independent variables. The weekly egg hatching rate was calculated based on the number of eggs and the number of juveniles appearing after hatching. If no significant differences were noted in this step, data were pooled for further analyses. A one-way ANOVA was conducted to determine if the concentration affected egg numbers, egg hatching rate, and molting frequency. After significance was assessed by ANOVA, Tukey's HSD post hoc comparisons were conducted using the LSMEAN option in SAS. All probability levels used for the statistical significance were $P < 0.05$.

The adult survival and juvenile reproduction between two controls (with acetone only and with distilled water only) were compared using the two-sample *t* test with two-tailed probability ($P = 0.05$). No observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) for reproduction were analyzed using analysis of variance (PROC ANOVA) followed by Dennett's test ($P = 0.05$) to set differences among test concentrations (USEPA 2005).

A concentration causing 50% of adult mortality (LC_{50}) and 50% reduction in egg hatching rate ($EC_{50\ =\ egg\ hatching\ rate}$) was determined by the Probit analysis, and a 50% reduction (EC_{50}) in juvenile production was estimated by fitting the data to the logistic model described by Haanstra et al. (1985):

$$y = c/[1 + \exp\{b \times (x - a)\}], \quad (1)$$

where y is the number of juveniles, x is the natural logarithm of the test concentrations, a is the natural logarithm of EC_{50}, b is the slope parameter, and c is the number of progeny per adult collembolan of the control. On the compressed soil, expected numbers of surviving adults and juvenile and egg production at each treated concentration of teflubenzuron ($\exp(x)$) could be estimated using the same method described in Eq. 1 by substituting the observed values for the eggs, juveniles, and adults.

Toxic contribution of teflubenzuron to each life parameter was estimated by calculating a ratio of juvenile production at the concentration to juvenile production at the control (no exposure) concentration using the data obtained from the compressed soil test. Since the juvenile productions are the outcome of the combination of adult survivals, egg production per adult, and egg hatching rate at the exposed concentration, toxic contribution rate (TCR) of each parameter can be determined by combining these three variables. Two assumptions were made in the model constructions: (1) the decrease in juvenile production with the teflubenzuron concentrations results from the toxic effects of teflubenzuron on adult survival, egg production, and egg hatching rate, and (2) the juvenile production is the result of the process by which surviving adults laid eggs, and the hatched eggs became juveniles.

Toxic contribution rate

The decrease in the number of juveniles due to the adult mortality (D_{adult}) at a specific concentration was equivalent to the number of juveniles that could be produced by dead adults in the control if the dead adults were assumed to be alive. Therefore, D_{adult} at an exposed concentration was calculated by the following equation:

$$D_{adult} = J_c - \left(\frac{J_c \times A_{conc}}{A_c}\right), \quad (2)$$

where J_c is the mean number of juvenile production in control, A_c is the mean number of surviving adults in control, A_{conc} is the estimated number of surviving adults at a specific concentration of teflubenzuron. A_{conc} can be estimated by substituting adult data in Eq. 1.

The decrease in the number of juvenile production due to decreases in egg production (D_{egg}) at a specific concentration is equal to the number of additional juvenile produced, if the surviving adults (A_{conc}) produce eggs, and the eggs hatch in the control. D_{egg} at an exposed concentration can be calculated by the following equation:

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$$D_{egg} = \left[\left(\frac{E_c \times A_{conc}}{A_c}\right) - E_{conc}\right] \times \frac{J_c}{E_c}, \quad (3)$$

where E_c is the mean number of egg production in control, E_{conc} is the estimated number of egg production at a specific concentration, and J_c/E_c is the egg hatching rate in control.

The decrease in the number of juvenile production due to decreases in egg hatching rate (D_{hatch}) at a specific concentration is equal to the number of additional juvenile produced, if produced eggs (E_{conc}) hatch in the control. D_{hatch} can be calculated as followed:

$$D_{hatch} = \frac{E_{conc} \times J_c}{E_c} - J_{conc}, \quad (4)$$

where E_{conc} is the estimated number of egg production, and J_{conc} is the estimated number of juvenile production at a specific concentration.

Toxic contribution of adult mortality (TA) could be defined as the ratio of D_{adult} to the difference in the number of juvenile production in the control (J_c) and at the exposed concentration (J_{conc}). The contributions of egg production reduction (TE) and egg hatching reduction (TH) could also be estimated using the same method applied for the TA. Thus, the contributions of TA, TE, and TH could be calculated by the following equations:

$$TA = \frac{D_{adult}}{(J_c - J_{conc})}, TE = \frac{D_{egg}}{(J_c - J_{conc})}, \text{and } TH = \frac{D_{hatch}}{(J_c - J_{conc})} \quad (5)$$

Each of TA, TE, and TH was calculated at the concentrations ranging from 0.26 mg/kg ($EC_{50\ =\ juvenile\ production}$) to 3.60 mg/kg ($LC_{50}$). Since each toxic contributor was estimated based on the juvenile production reduction at the treated concentration, the sum of the three contributors should be 1.

Results

In the controls of the compressed soil test, acetone treatment did not affect adult survival, egg production, and juvenile production when compared with the water-treated control ($t = -1.00$, $P = 0.36$ for adult survival; $t = -0.45$, $P = 0.67$ for egg production; $t = -1.16$, $P = 0.29$ for juvenile production). No adult mortality was observed in both controls. The mean number of egg and juvenile reproduction in the acetone control was 162.0 ± 24.1 and 99.2 ± 12.2 per plate, respectively. The same was true in the controls of the ISO standard method. Adult survival and juvenile production were not significantly affected ($t = -0.4$, $P = 0.70$ and $t = 0.41$, $P = 0.69$, respectively). Mean adult mortality and juvenile production for the 28-day exposure in the acetone control was 12.5 ± 0.5% and 58.5 ± 10.4 per container, respectively. These results indicate that the use of acetone as a solvent for teflubenzuron was appropriate for the toxicity study.

Adult survival, egg production, and juvenile production in the compressed soil

The effects of teflubenzuron on adult survival (LC_{50}, mg/kg) and egg and juvenile reproduction (EC_{50}s, mg/kg) and the corresponding NOEC and LOEC concentrations were calculated weekly for 28 days, with an exception of the surviving adults (Fig. 1 and Table 1). For adult survival, weekly cumulative LC_{50} values were estimated because the same adults were exposed to teflubenzuron and observed in a weekly manner. The LC_{50} values decreased with the increased exposure period from 6.97 in the third week to 3.60 in the fourth week. However, the LC_{50} values for the first 2 weeks could not be determined because all mortality values observed were below 50%, even when exposed to the highest concentration tested (16 mg/kg).

The egg production decreased with increased concentrations ($F = 46.82$; df = 7, 39; $P < 0.0001$), and no eggs were produced beyond the concentration of 4 mg/kg. Weekly concentration-response curves showed that there were no significant differences between egg production among the weeks ($F = 0.44$; df = 3, 159; $P > 0.98$), ranging from 0.45 to 0.65 mg/kg (Table 1 and Fig. 2S). Thus, the weekly data were pooled to estimate the overall effect on reproduction. The pooled $EC_{50\ =\ egg\ production}$ was estimated to be 0.57, and the corresponding NOEC and LOEC were 0.25 and 0.50 mg/kg, respectively.

Egg hatching rate was calculated based on the number of eggs and the number of juveniles appearing after hatching.

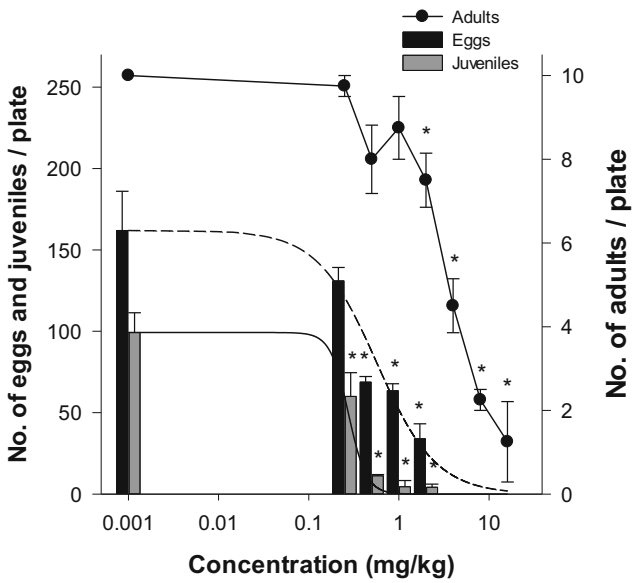

Fig. 1 The number of surviving adults (●), eggs (black bars), and juveniles (gray bars) of *Yuukiaura szeptyckii* after a 28-day exposure to teflubenzuron in the compressed soil test. Predicted egg (dashed line) and juvenile (solid line) numbers were fitted by Eq. (1)

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Table 1 Estimation of weekly LC_{50} for adults and EC_{50}s for egg and juvenile reproduction (mg/kg in soil) of *Yuukianura szeptyckii* with 95% confidence limit after 28-day exposure to various teflubenzuron concentrations in compressed soil media. NOEC and LOEC for egg and juvenile production were determined using the Dunnett test (5% level). NOEC for juvenile production could not be determined because all concentrations tested significantly reduced reproduction compared with the control

Week	LC_{50}[a]	$EC_{50\ =\ egg\ production}$	NOEC	LOEC	$EC_{50\ =\ juvenile\ production}$	NOEC	LOEC
1	–[b]	0.61 (0.48–0.78)	0.25	0.50	0.22 (0.20–0.24)	–	0.25
2	–	0.45 (0.28–0.73)	0.25	0.50	0.20 (0.17–0.24)	–	0.25
3	6.97 (5.67–8.59)	0.65 (0.45–0.95)	0.50	1.00	0.21 (0.17–0.26)	–	0.25
4	3.60 (2.90–4.50)	0.55 (0.32–0.94)	0.25	0.50	0.24 (0.23–0.27)	–	0.25
Pooled	3.60 (2.90–4.50)	0.57 (0.48–0.67)	0.25	0.50	0.26 (0.23–0.32)	–	0.25

[a] Cumulative LC_{50} value was estimated because same adults were observed in a weekly manner

[b] The LC_{50} could not be determined because the mortality values observed were below 50%, even when exposed to the highest concentration of teflubenzuron tested (16 mg/kg)

Weekly egg hatching rates did not differ significantly between the weeks ($F = 0.07$; df = 3, 78; $P > 0.98$) (Fig. 2S), and thus the data were pooled to estimate the overall hatching rate according to the concentrations. The hatching rate decreased significantly by 61 ± 2.0, 46 ± 8.0, 16 ± 1.0, 7 ± 5.0, and 13 ± 2.0% as the exposure concentration increased at 0, 0.25, 0.5, 1, and 2 mg/kg ($F = 16.38$, df = 4, 78, $P < 0.0001$). $EC_{50\ =\ egg\ hatching}$ was estimated to be 0.41 mg/kg.

The juvenile production also decreased with increased concentrations ($F = 34.05$; df = 7, 39; $P < 0.0001$), but the slopes of the dose-response curves decreased more rapidly as the concentration increased compared to the curves observed in the egg production (Fig. 2S). These results indicated that the juvenile reproduction was strongly affected by the subsequent effects of egg hatching on egg production.

Mean number of molt per adult individual per week decreased gradually with increasing teflubenzuron concentrate from 1.10 ± 0.10 in the control to 0.80 ± 0.15 during the 28-day exposure (Fig. 2). The significant differences were observed only at > 4 mg/kg of teflubenzuron in comparison with controls ($F = 12.76$; df = 7, 39; $P < 0.01$), indicating that the effect of teflubenzuron treatments on the adult growth of *Y. szeptyckii* is minimal at lower concentrations (< 4 mg/kg).

Toxic contribution rate

The toxic contribution of TA increased exponentially with increased concentrations, ranging from 0.12 to 0.50, but TH decreased greatly with the concentrations from 0.65 to 0.80 (Fig. 3). TE increased gradually until 1.50 mg/kg teflubenzuron and decreased subsequently with relatively less variations as the concentration changed, ranging from 0.32 to 0.54. TCR at 0.26 mg/kg ($EC_{50\ =\ juvenile\ production}$) was 0.04, 0.32, and 0.64 for TA, TE, and TH, respectively. At 0.57 mg/kg ($EC_{50\ =\ egg\ production}$), TCR of each contributor was 0.06, 0.54, and 0.40, respectively. At 3.60 (LC_{50}), contribution of TH was small (0.08), but the contributions of TA and TE were similar (0.50 and 0.42). These modeling analyses showed that TCRs of teflubenzuron to *Y. szeptyckii* changed with the concentrations exposed. At lower concentrations (< 0.3 mg/kg), a negative impact of teflubenzuron on egg hatching rate was a main contributor to the total toxicity, but negative impacts on adult survival and egg production were the main contributors at the higher concentrations (> 2.0 mg/kg).

Toxicity comparison between the compressed soil test and the ISO standard method

To evaluate and compare the performance of the compressed soil test to the ISO standard method, adult survival (LC_{50}) and juvenile reproduction (EC_{50}), which were commonly used endpoints in both methods, were compared. The estimated LC_{50} in the ISO standard method was 0.90 mg/kg, which was four times lower than that observed in the compressed soil test (3.60 mg/kg) (Tables 1 and 2). The similar difference

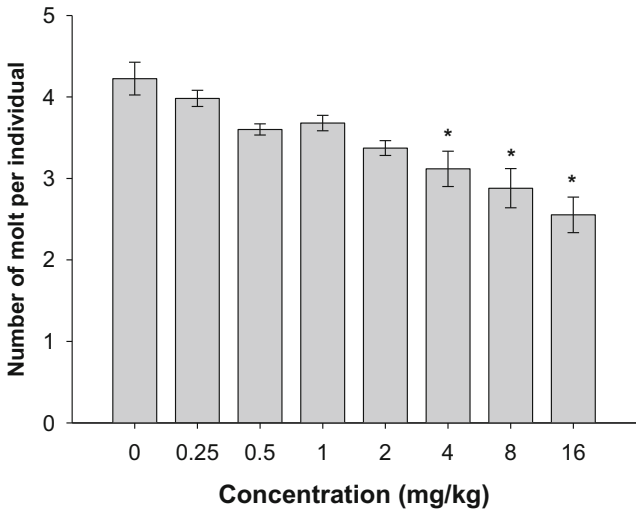

Fig. 2 Changes of mean molt numbers per adult *Yuukianura szeptyckii* exposed to various teflubenzuron concentrations in the compressed soil. Weekly data were pooled because there were no significant differences in molt numbers between weeks ($F = 0.71$; df = 7, 159; $P < 0.55$)

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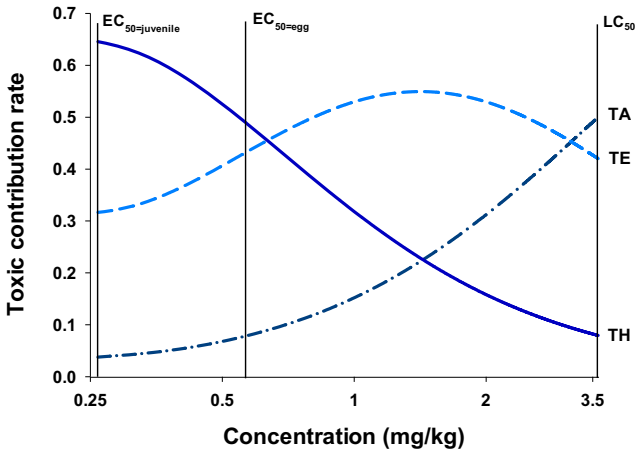

Fig. 3 Calculation of toxic contribution rate (TCR) relative to decrease in juvenile production caused by adult mortality (TA), egg production reduction (TE), and egg hatching rate reduction (TH) within the range of 0.26 mg/kg ($EC_{50\ =\ juvenile\ production}$) to 3.60 mg/kg ($LC_{50}$). Each TCR value was calculated using Eq. 5

was observed in the EC_{50} values (0.26 mg/kg and 0.07 mg/kg).

Degradation of teflubenzuron

Degradation of teflubenzuron in the soil was determined weekly within a 28-day timeframe (Fig. 3S). No significant degradation differences were observed over the 28-day period (relative standard deviation (RSD) = 9.43%), confirming its persistence in soil. The high persistence of teflubenzuron in the soil is expected due to its adsorption characteristics to various organic and non-organic soil components as reported by Heupt (1984).

Discussion

The current ISO 11267 protocol (ISO 1999) for the soil toxicity test with collembolans has been widely used to obtain consistent and comparable summary results for the chemical risk assessment such as LC_{50} (adult survival) and EC_{50} (juvenile production). However, the ISO test needs to include more realistic and ecologically relevant endpoints for a better understanding of how chemicals affect target organisms, especially for the chemicals such as IGRs that have a variety of toxic mechanism. Since the toxic effects of IGRs are highly variable depending on the species and developmental stage, previous laboratory toxicity tests of IGRs with non-target species have been evaluated with various endpoints, including adult survival, egg production, and larval development to infer the risks associated with exposure in real environments (Campiche et al. 2006; Darvas and Polgar 1998; Lee et al. 2018; Schneider et al. 2004). These previous reports clearly indicate the limitations of the current Collembola reproduction test proposed by ISO 11267 (ISO 1999).

Table 2 Estimation of LC_{50} for adults and EC_{50} for juvenile reproduction (mg/kg in soil) of *Yuukianura szeptyckii* with 95% confidence limit after 28-day exposure to various teflubenzuron concentrations in the ISO standard test method. LOEC for juvenile production was determined using the Dunnett test (5% level). NOEC could not be determined for juvenile production because all concentrations tested in this study significantly reduced reproduction compared to the control.

LC_{50}	EC_{50}	NOEC	LOEC
0.90 (0.70–1.10)	0.07 (0.05–0.09)	–	0.06

The EC_{50} for juvenile production of *Folsomia candida*, a standard species of ISO standard guideline, was reported as 0.05 mg/kg (Campiche et al. 2006). A similar value (EC_{50} = 0.07 mg/kg) was observed in this study when *Y. szeptyckii* was evaluated by the ISO method (Table 2). It is reported that an initial worst-case predicted environmental concentration (PEC_i) of teflubenzuron was 0.06 mg/kg (EU 1991; Campiche et al. 2006). Considering PEC_i and the two EC_{50} values, teflubenzuron is considered to have a negative effect on the collembolan population at the environmentally relevant level of concentration.

When comparing the ISO and the compressed soil test methods with the two common endpoints (adult survival and juvenile reproduction), the ISO method showed higher toxicities than the compressed soil test (Tables 1 and 2). This difference in toxicity seems to be due to differences in exposure pathways of teflubenzuron. In the compressed soil test, the collembolans could not burrow into the soil, and all life stages of eggs, juveniles, and adults were present on the soil surface. However, the ISO method allowed the collembolans to burrow into the soil. Thus, the collembolans in the compressed soil test were considered to be less exposed to teflubenzuron through dermal contact than those in the ISO. Son et al. (2011) reported that chemical toxicity was closely related to the magnitude of exposure to the toxic chemical through dermal contact and showed that cadmium toxicity to collembolans decreased significantly as soil compaction increased. Comparing the LC_{50} and $EC_{50\ =\ juvenile\ production}$ of ISO standard method to those of the compressed soil test, the ISO method has always produced the toxicity values that are four times higher than the compaction method (Tables 1 and 2), indicating that there was no difference in the trends of the two endpoints between the test methods but only the magnitude of the values. Therefore, the toxicity values examined by the compressed soil method can be easily and reliably converted to the toxic values of the ISO method.

The compressed soil test method used in this study is proven to be suitable for assessing the toxic effects of teflubenzuron with all crucial life cycles of collembolans,

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including the adult survival, egg production, egg hatching rate, and juvenile production. The juvenile production was the most sensitive endpoint (0.25 mg/kg) to teflubenzuron, and the others were in the order of the egg hatching rate (0.41 mg/kg), egg production (0.57 mg/kg), and adult survival (3.52 mg/kg). These results were similar to other reports that the juvenile production was the most sensitive endpoint in the ISO Collembola reproduction test (ISO 1999) performed on IGRs (Campiche et al. 2006; Lee et al. 2018) and heavy metals (Son et al. 2007). Interestingly, the egg hatching rate, which could not be determined in the ISO method, was the second most sensitive to teflubenzuron exposure in the compressed soil method. According to the toxicology studies with other insect species, a decrease in egg hatching rate is known to be the most representative symptom of exposure to teflubenzuron (Abo-Elghar et al. 2004; Acheuk et al. 2012).

The reproduction of collembolans is considered to be a sensitive and ecologically relevant parameter to assess chemical toxic effects; therefore, the toxic contribution rate (TCR) of teflubenzuron on each life history parameter was calculated based on the effects on the juvenile production. The toxic contributor TH decreased with teflubenzuron concentrations, but the TA increased. The TE had an intermediate value in the concentration range (Fig. 3). These different contributions of each TCR with concentrations might be due to the difference in sensitivities to teflubenzuron in each life history parameter. Specifically, the sensitivity decreased with concentration in order of egg hatching rate, egg production, and adult mortality. Grime (1989) reported that the sensitivity of individuals within the population to toxicants is dependent on developmental stages. Assuming the field conditions with mixed life stages of collembolans, the population size at a low concentration could be controlled by the decrease in egg hatching rate and by adult mortality at high concentrations. To the best of our knowledge, this is the first study to evaluate the relation of pesticide concentrations to TCRs of negative impact on various life history parameters. There are some studies evaluating differences of the toxic contribution between chemical species to organisms (He et al. 2015; Zhang et al. 2016), but very few studies have examined toxic contributions of the same chemicals to the life history parameters of the same animal species. Our study suggests that toxicity sensitivity of each life history parameter and exposure concentration are important factors when assessing the toxic effect of insect growth regulators, because, unlike the laboratory conditions, collembolan populations in the soil ecosystem are composed of individuals with various developmental stages.

Conclusion

In this study, the toxic effects of teflubenzuron on *Yuukianura szeptyckii*, a collembolan species, were evaluated using the ISO standard method (ISO 1999) and the compressed soil method. Comparing the two test methods with two common endpoints (adult mortality and juvenile production), the toxicity values differed only in magnitude, but the compressed soil method had the advantage of observing additional endpoints such as egg production, egg hatching rate, and molting frequency. The toxic contribution rate (TCR) of negative impacts on each life history parameter to the juvenile production was modeled and calculated based on the toxic effects measured in the compressed soil method. TCRs were changed in a concentration-dependent manner. At lower concentrations of teflubenzuron, egg hatching reduction was a main contributor to the juvenile reduction, but adult mortality and egg production reduction were the main contributors at higher concentrations. These results demonstrated that the sensitivity of each life history parameter to chemicals and exposure concentration should be taken into account together when assessing the ecological risk of chemicals because the collembolan populations in the soil ecosystem are composed of various developmental stages. In this respect, the compressed soil test is a suitable and ecologically relevant method for understanding toxicity characteristics of toxicant related to life history parameters of collembolans. The ISO test method and the compressed soil method can be applied complementary to the ecotoxicological risk assessments of chemicals. The ISO method can be used for toxicity comparison studies with others, and the compressed soil test can be applied to evaluate the contributions of each life history parameter of collembolans to the total toxicities in the soil ecosystem.

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