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Environmental hazards of nanopesticides to non-target soil species - commercial nanoformulation versus its active substance (Karate Zeon® and lambda-cyhalothrin)

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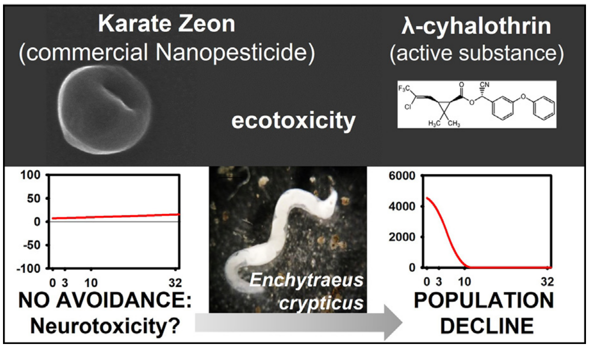
HIGHLIGHTS

- The commercial insecticide Karate Zeon contains micro and nanocapsules.
- Effects of Karate Zeon and its active substance λ-cyhalothrin were assessed.
- Karate Zeon and λ-cyhalothrin similarly toxic: hatching, survival, reproduction.
- No avoidance was probably due to neurotoxicity in *E. crypticus*.
- From the full life cycle test, juvenile stage was the most affected.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanopesticides (Npes) carry the potential of increased efficacy while reducing application rates, hence increasing agricultural productivity in a more sustainable way. However, given its novelty, the environmental risk assessment of these advanced materials is mostly absent. In the present study we investigated the ecotoxicity of a commercial insecticide, with reported nanofeatures, Karate Zeon®, and compared it to its active substance lambda-cyhalothrin. It is hypothesised that the use of the nanopesticide Karate Zeon® poses lower risk to enchytraeids than its active substance. The standard non-target soil invertebrate *Enchytraeus crypticus* was used, and exposure was done in LUFA 2.2 soil in 4 tests (endpoints: days): avoidance test [avoidance behaviour: 2 days], OECD standard reproduction test [survival, reproduction plus adults' size: 28 days] and its extension [total number organisms: 56 days], and Full Life Cycle (FLC) test [hatching and juveniles' size: 13 days; survival, reproduction and adults' size: 46 days]. Results showed that enchytraeids did not avoid Karate Zeon® nor its active substance lambda-cyhalothrin, which could be due to neurotoxicity. There was no indication of increased toxicity with prolonged exposure (46, 56d) compared to the standard (28d) for neither of the materials, being overall equally toxic in terms of hatching, survival, and reproduction. The FLCt results indicated that the juvenile stage was the most sensitive, resulting in higher toxicity for the adult animals when exposed from the cocoon stage. Although toxicity was similar between Karate Zeon and lambda-cyhalothrin, different patterns of uptake and elimination cannot be excluded. The benefits of using Karate Zeon will rely on reduced application rates.

1. Introduction

Currently, one of the greatest challenges is to feed a growing population while keeping environmental sustainability. Nanopesticides (Npes) are

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more environmentally sustainable alternatives to conventional pesticides, due to their increased effectiveness and targeted delivery, e.g. allowing controlled release of the active substance (Chariou et al., 2020; Nuruzzaman et al., 2016; Scott-Fordsmand et al., 2022; Wang et al., 2022; Xu et al., 2022). Hence, the environmental footprint can be reduced via a reduction of the application rate, while increasing food safety (less pesticides residues) and production. However, given the novelty, the environmental safety and sustainability assessment of Npes are still lacking and represent a concern (Grillo et al., 2021a, 2021b; Kah et al., 2021; Walker et al., 2018; Xu et al., 2022). Further, it is not always clear which regulation applies to Npes and certain products are commercialised without a fully clear description of composition.

There is intensive research being devoted to the development of new Npes, partly by proposing new nano-carriers for known active substances (e.g. (Ding et al., 2023; Sun et al., 2022; Wan et al., 2022; Xiao et al., 2022)) or plant extracts (e.g. (Iqbal et al., 2022; Oliveira et al., 2019)).

There are also already currently marketed Npes products based on for instance nanopolymers (Walker et al., 2018). However, the identification of Npes in the marketed products is not transparent, with size being absent from the label or unclear for the consumer.

The labelling of presence of engineered nanomaterials is known to be legally required for example for foods [labelling provisions (Regulation (EU) 1169/2011)]. This dissonance is in part due to the lack of an internationally accepted definition of Npes, leading to the adoption of different size ranges and different limits for the fraction of nanosized particles by the regulatory agencies (Grillo et al., 2021b; Kah et al., 2021; Wang et al., 2022). The available literature data show that most Npes would not fit within the 100 nm size distribution definition, yet, some of the nanomaterial (NM) related properties remain and should require attention within the guidance for risk assessment of NMs applied to food and agriculture (Hardy et al., 2018). Although this is more of a regulatory matter concern, it hampers the research drive and related awareness.

For example, Kocide®3000, a commercial fungicide based on cooper hydroxide, is a Npe or nanoenabled pesticide (Li et al., 2019), although such information is not apparent in the label. Kocide is one of the most studied Npes in literature in terms of ecotoxicological effects, and significant toxicity has been reported for both aquatic, e.g. zebrafish, daphnia, and terrestrial species, e.g. collembolans, isopods (Aksakal and Sisman, 2020; Morgado et al., 2022; Neves et al., 2019; Wang et al., 2021). A lambda-cyhalothrin based insecticide, Karate Zeon®, is another example of a commercial Npe, it is labelled as a capsule suspension formulation, containing both microcapsules and nanocapsules (~ 200 nm) (Meredith et al., 2016). Karate Zeon has shown to be more lethal to zebrafish embryos than it's active substance (LC50 = 150 and 273 μg/L for Karate Zeon and lambda-cyhalothrin, respectively) (Meredith et al., 2016). The Npe was tested as total and fractionated formulation (with separation of the nano-fraction and micro-fraction) without significant differences between these (Meredith et al., 2016). The higher toxicity of Karate Zeon, compared to its active substance, to zebrafish embryos, was even higher in terms of the sub-lethal endpoints: embryonic tremoring (24 h EC50 = 37 and 66 μg/L for Karate Zeon and lambda-cyhalothrin, respectively) and pericardial edema (96 h EC50 = 31 and >80 μg/L for Karate Zeon and lambda-cyhalothrin, respectively) (Meredith et al., 2016). Another study using fractionated Karate Zeon showed higher toxicity of the smaller fraction (450 nm), compared to the larger fraction (750 nm) and lambda-cyhalothrin to the freshwater crustacean *Ceriodaphnia dubia* (EC50 = 0.18 μg/L, 0.57 μg/L, and 0.65 μg/L for the smaller fraction, the larger fraction and the active substance, respectively).

As exemplified by the mentioned studies on Kocide and Karate Zeon, two marketed pesticides with nano characteristics were toxic to environmentally relevant non-target species. However, the comparison to the active substance is not always available, and when available it shows that differences (higher or lower toxicity) sometimes depended on the test species, which is of importance. A recent review (Wang et al., 2022) indicated that Npes exhibit less toxicity to non-target organisms when compared to the non-nano analogues. However, a very limited number of the studies considered referred to environmentally relevant species, such as aquatic or soil living invertebrates, often in the first line of exposure from pesticides application run-offs. Further, the vast majority of studies considered (Wang et al., 2022) concerns bare nanoparticles (e.g. Ag and Cu) with biocidal properties or laboratory synthesized formulations to be used as NPes, with only a very small fraction of the literature referring to marketed products.

This scarcity of information regarding assessment of hazards to non-target species of commercial Npes or nanoenabled pesticides, shows the urgent need to fill in the gap, particularly to soil living invertebrates.

The aim of the present study was to evaluate the effects of the commercial insecticide Karate Zeon®, which is based on a capsule suspension formulation with reported nanometer size capsules (Meredith et al., 2016; Paz-Trejo et al., 2022; Slattery et al., 2019), and compare it to its active substance lambda-cyhalothrin. Exposure was done using the soil invertebrate *Enchytraeus crypticus* (Oligochaeta) and the natural standard LUFA 2.2 soil. Enchytraeids are model species in soil ecotoxicology (OECD 220, 2016), with a wide distribution in soils worldwide where they contribute to the improvement of the pore structure of the soil and, indirectly, to the degradation of organic matter (Rombke and Moser, 2002). Effects were assessed covering various endpoints and life-stages as based on: avoidance test [2 days: avoidance behaviour], OECD standard reproduction test (OECD 220, 2016) [28 days: survival, reproduction and adults' size] and its extension (Ribeiro et al., 2018) [56 days: total organisms], and Full Life Cycle test (Bicho et al., 2015b) [13 days: hatching and juveniles' size; 46 days: survival, reproduction and adults' size]. We here hypothesize that the use of the nanopesticide Karate Zeon® poses lower risks to enchytraeids, when compared to its active substance, lambda-cyhalothrin.

2. Materials and methods

2.1. Test organism

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions at the final concentrations of 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$, under controlled conditions of temperature (19 ± 1 °C) and photoperiod (16:8 h light:dark). The cultures were fed with ground autoclaved oats twice per week.

2.2. Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The soil main characteristics are: pH (0.01 M $CaCl_2$) = 5.6, organic matter = 1.77 %, CEC (cation exchange capacity) = 8.5 meq/100 g, WHC (water holding capacity) = 43.3 %, grain size distribution of 10.6 % clay (<0.002 mm), 15.0 % silt (0.002–0.05 mm), and 74.4 % sand (0.05–2.0 mm).

2.3. Test materials and characterization

Karate Zeon® (100 g/L or 9.5 % w/w lambda-cyhalothrin, Syngenta®) and its active substance lambda-cyhalothrin (PESTANAL®, analytical standard, Merk) were purchased.

Karate Zeon was characterized by Dynamic Light Scattering (DLS), Zeta-Potential and Scanning/Transmission Electron Microscopy (STEM). DLS measurements were carried out with a Zeta-Sizer Malvern Instrument (Zetasizer Nano ZS, Malvern Ltd., UK) in backscattering mode to determine hydrodynamic size and charge (Zeta-potential). All measurements were performed in auto-mode at 25 °C, with 3 consecutive measurements for each sample, using the same samples as to spike the soil. The morphology of Karate Zeon capsules was analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), using a JEOL 2200FS HR-TEM instrument (JEOL, Tokyo, Japan) operating at 200 kV. The sample was prepared by dropping (twice) 20 μL of the formulation (50 mg/L) on a carbon-coated Cu grid and drying, at room temperature, before imaging.

2.4. Spiking procedures

The tested concentrations were 0, 0.32, 1, 3.2, 10, 32 mg lambda-cyhalothrin/kg soil dry weight (DW) for both test substances (Karate Zeon and pure active substance lambda-cyhalothrin).

Karate Zeon is an aqueous concentrated suspension, thus it was serially diluted from a stock solution (0.32 mL in 100 mL of water, corresponding to the 32 mg active substance/kg soil), using MQ water, to obtain the desired test concentrations. The spiking followed the guidelines for nanomaterials (OECD, 2012). Briefly, the prepared suspensions were added to the pre-moistened soil (as 10 mL solution per 100 g of soil) to reach 50 % of soil's maximum WHC, with each replicate prepared individually (to ensure total raw amounts of the tested material). The soil was homogeneously mixed and left to equilibrate for 1 day prior to the start of the tests.

Lambda-cyhalothrin was dissolved in acetone, due to its low solubility in water, and serially diluted to the desired test concentrations, homogeneously mixed into the batches of soil (per concentration) and left to evaporate in a fume hood for 24 h. A solvent (acetone) control was prepared in parallel, adding acetone alone to the soil, in the equivalent volume as that used for the concentration range. After 24 h, the soil was moistened (with deionised water) until 50 % of soil's maxWHC and introduced in each test vessel. The tests started immediately thereafter.

2.5. Ecotoxicity tests

Toxicity assessment was done based on a series of 3 different test types, covering various endpoints and life-stages. The test procedures are detailed bellow in the following order i) avoidance tests (duration: 2 days; endpoint: avoidance behaviour), ii) OECD standard reproduction tests (duration: up to 56 days; endpoints: survival, reproduction and adults' size (28 days), and total organisms (56 days)), and iii) Full Life Cycle tests (duration: up to 46 days; endpoints: hatching and juveniles' size (13 days), survival, reproduction and adults' size (46 days).

2.5.1. Avoidance tests

Avoidance tests were performed following the earthworm avoidance test guideline (ISO 17512-1, 2008), using *E. crypticus* with adaptations as described in Bicho et al. (2015a). In short, plastic containers (2.5 × 6.5ø cm) with one removable plastic divider were used; each replicate contained 50 g of soil (25 g each side), this being the control and spiked soil. After soil placement, the wall was gently removed, and ten adult organisms (with clitellum) were placed on the contact line of the soils. Boxes were covered with a lid (containing small holes) and kept, for 48 h, at 20 ± 1 °C and a photoperiod of 16:8 h (light:dark). Five replicates per treatment were used. At the end of the test period, the divider was again inserted in the separation line between the two soils and each side of the box was independently searched for enchytraeids. For the Karate Zeon test, the control consisted of moist (50 % maxWHC) LUFA 2.2 soil and for the lambda-cyhalothrin test, the control consisted of the solvent control; an additional solvent control versus water control was performed to assess the possible effects of acetone.

2.5.2. Enchytraeid reproduction tests: standard and extension

The tests followed the standard guideline for the Enchytraeid Reproduction Test (ERT, 28 days) (OECD 220, 2016), plus the OECD extension, as described in, e.g., Ribeiro et al. (2018). In short, the test was extended for an additional 28 days (56 days in total) and extra monitoring sampling times were taken at days 7, 14, 21, (28) and 56. Endpoints included survival for all sampling periods, reproduction at days 28 and 56, i.e., number of juveniles and population, respectively, and size at day 28. Four replicates per treatment were carried out, except at days 7, 14 and 21 (1 replicate). At test start, ten synchronized age organisms (18–20 days old after cocoon laying) were introduced in each test vessel with moist soil (7, 14, 21, and 28-days exposure: Ø4 cm vessel, 20 g of soil, and 56 days exposure: Ø5.5 cm vessel, 40 g of soil) and food supply (22 ± 2 mg, autoclaved rolled oats). The test ran up to 56 days at 20 ± 1 °C and 16:8 h photoperiod. Food (11 ± 1 mg: until day 28, and 33 ± 3 mg: from day 28 to 56) and water (based on weight loss) was replenished weekly. On sampling days 7, 14, 21, and 28, adults were carefully removed from the soil and counted (survival). The juveniles were counted at day 28 and 56 using a stereo microscope, to assess reproduction. After being fixed for 24 h with ethanol and Bengal rose (1 % in ethanol), soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. For the replicates that continued until day 56, adults were carefully removed from the soil at day 28. The adult organisms collected at day 28 were photographed after staining with Bengal rose, and size (length, mm) was assessed using the software ImageJ (v.1.52a).

2.5.3. Full life cycle tests

A reduced version of the full life cycle test (FLCt), as described by Bicho et al. (2015b) was performed. Assessed endpoints included hatching success and juveniles' length (day 13), survival, reproduction, and adults' length (day 46). In short, the test starts with cocoons (1–2d old) selected from synchronized cultures. Ten cocoons were introduced in each test vessel (ø 40 mm, 7.5 cm height) containing 20 g of moist soil (50 % maxWHC) and the test ran at 20 ± 1 °C with a 16:8 h (light:dark) photoperiod. Four replicates per treatment were done, including controls (water and solvent control for lambda-cyhalothrin). Food (6 mg autoclaved ground oats) was added for the first time at day 13 and then replenished weekly together with water content (based on weight loss). At each sampling time point, the respective replicates were processed, and organisms were counted (using a stereo microscope) following the method described above. A subsample of the organisms in each replicate (n = 10) was photographed for size measurement (length, mm), as described above.

2.6. Data analysis

Avoidance was calculated as the percentage of worms that avoided the treated soil in the test container from the total number of worms in that container. The mean percentages of net responses (NR) were calculated as follows:

$$NR = ((C - T)/N) \times 100,$$

where C is the number of organisms observed in the control soil, T is the number of organisms observed in the test soil and N is the total number of organisms per replicate. A positive (+) NR indicates avoidance, and a negative (−) NR indicates a non-response (or attraction) to the chemical.

For the lambda-cyhalothrin tests, the controls (water and solvent) were compared using the *t*-test, at a significance level of 0.05. One way analysis of variance (ANOVA), followed by the post-hoc Dunnett's method (for multiple comparisons), was used to assess the differences between test treatments and control, at a significance level of 0.05 (SigmaPlot 14.0). Effect concentrations (ECx) were calculated, for the various endpoints, modelling data to logistic or threshold sigmoid 2 parameter regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program (TRAP 1.30a) software. Avoidance data were inverted to apply the regression models.

3. Results

3.1. Materials characterization

Karate Zeon suspensions are highly polydisperse, with large agglomerates when in aqueous media. The average hydrodynamic sizes were above 2000 nm, for all the tested concentrations (Table S1), thus with no apparent size-dose relationship. Smaller peaks (in the range of 100 nm) were occasionally identified, and particles can be found in that range as can be depicted in Fig. 1. The surface charge of Karate Zeon suspensions was always below −36.5 mV, decreasing with increasing concentrations (Table S1). SEM/TEM pictures (Fig. 1) show that the capsules are spherical in shape and are present in the nanometer size range (ca. 100 nm, Fig. 1) as well as in larger sizes (Fig. 1C). The TEM picture (Fig. 1A) shows a less

Table 1

Summary of the effect concentrations (ECx, with 95 % confidence intervals - CI), expressed as mg lambda-cyhalothrin per kg soil dry weight, for *Enchytraeus crypticus* exposed to Karate Zeon and its active substance lambda-cyhalothrin, in LUFA 2.2 soil. The models used are Threshold sigmoid 2 parameters (Thres2P) or Logistic 2 parameters (Log2P). S: slope; y0: top point; n.e.: no effect. Grey values: should not be assumed directly, mere estimation (high CI and low r^2).

Test material/endpoint	Time (days)	EC20 (95 % CI)	EC50 (95 % CI)	EC80 (95 % CI)	Model & parameters
Karate Zeon					
Avoidance	2	13.3 (−18–44)	115.7 (−135–367)	218.1 (−272–706)	Log2P; S:0.003; y0:112; r^2:0.1
Survival	28	5.8 (5–7)	8.2 (7–9)	9.8 (9–11)	Thresh2P; S:0.2; y0:9.8; r^2:0.97
Reproduction	28	2.8 (1–5)	5.2 (3–8)	7 (2 − 12)	Thresh2P; S:2.1; y0:692; r^2:0.79
Size (adults)	28	10.7 (5–17)	13.0 (−13–38)	15.2 (−30–60)	Log2P; S:0.2; y0:7.5; r^2:0.3
Total organisms	56	2.8 (1–4)	5.3 (3–7)	7 (3 − 11)	Thresh2P; S:0.2; y0:4643.9; r^2:0.84
Hatching	13	7.9 (0–16)	9.8 (9–11)	11.8 (5–18)	Log2P; S:0.2; y0: 30.8; r^2: 0.85
Size (juveniles)	13	n.e.	n.e.	n.e.	
Survival	46	2.3 (1–4)	6.2 (5–8)	9 (7–11)	Thresh2P; S:0.1; y0:28.9; r^2:0.9
Reproduction	46	0.7 (1–2)	3.5 (2–5)	6.3 (3 − 10)	Log2P; S:0.1; y0:2360.2; r^2:0.74
Size (adults)	46	31.8 (23–41)	45.9 (25–67)	59.9 (24–92)	Log2P; S:0.025; y0:7.7.2; r^2:0.3
Lambda-cyhalothrin					
Avoidance	2	30.8 (21–41)	41.8 (7–76)	52.8 (−17–122)	Log2P; S:0.006; y0:39.6; r^2:0.7
Survival	28	9.8 (8–11)	11.6 (0 − 23)	13.3 (−12–39)	Log2P; S:0.2; y0:9.7; r^2:0.97
Reproduction	28	4.2 (2–6)	6.3 (4–8)	7.9 (5–11)	Thresh2P; S:0.2; y0:695; r^2:0.85
Size (adults)	28	8.6 (6–11)	14.3 (9–20)	18.5 (10–27)	Thresh2P; S:0.06; y0:7.3; r^2:0.003
Total organisms	56	1.5 (0–3)	3.8 (2–6)	6.2 (2 − 10)	Log2P; S:0.1; y0:4676.9; r^2:0.73
Hatching	13	4.8 (3–6)	8.4 (7–10)	12.1 (10–14)	Log2P; S:0.1; y0:32.3; r^2:0.9
Size	13	7.7 (6–9)	11.5 (10 − 13)	15.3 (12–18)	Log2P; S:0.1; y0:1.32; r^2:0.8
Survival	46	0.8 (0–2)	3.9 (3–5)	6.2 (4–9)	Thresh2P; S:0.1; y0:30.1; r^2:0.87
Reproduction	46	1.6 (1–3)	3.9 (2–5)	6.1 (3–9)	Log2P; S:0.2; y0:2341.1; r^2:0.87
Size (adults)	46	13 (−1–27)	17.2 (−10–44)	21.3 (−19–62)	Log2P; S:0.1; y0:7.8; r^2:0.007

dense central area consistent with the presence of a cavity inside the capsule, where the active substance is loaded.

3.2. Ecotoxicological tests

For all the tests performed with lambda-cyhalothrin, there were no significant differences between the controls: control (unspiked soil, water) versus control-acetone, thus controls were pooled for the graphs and statistical analysis.

3.2.1. Avoidance tests

The validity criteria were fulfilled, i.e., <20 % mortality and homogeneous distribution (no avoidance) in controls. There was no significant avoidance of the spiked soil (Fig. 2), even though there was increased avoidance response at the highest tested concentration.

Please note that the estimated EC (Table 1) should not be assumed directly given the lack of actual higher impact measured within the experiment (see discussion for further details).

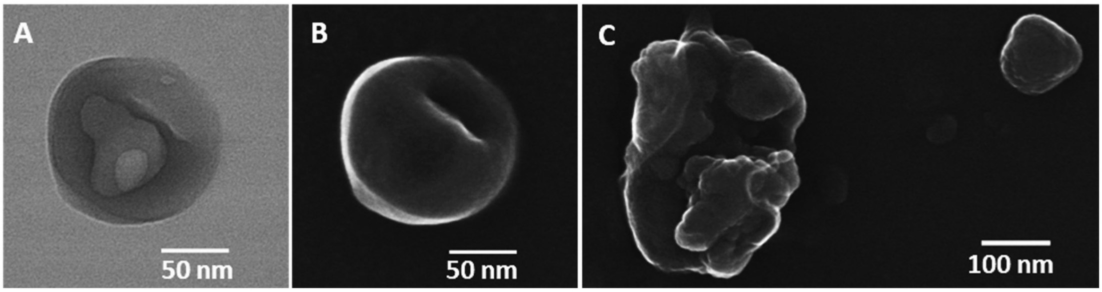

Fig. 1. High resolution electron microscopy (EM) pictures of Karate Zeon capsules, in A) Transmission (TEM) mode and B, C) Scanning (SEM) mode.

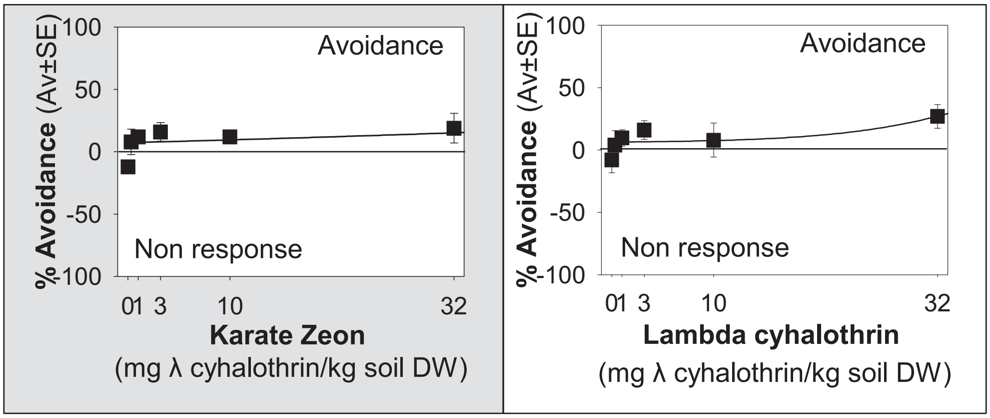

Fig. 2. Results in terms of avoidance response of *Enchytraeus crypticus* exposed for 48 h, in LUFA 2.2 soil, to Karate Zeon and Lambda-cyhalothrin (Karate Zeon's active substance). Data is expressed as average ± standard error (Av ± SE). Lines represent the models fit to data.

3.2.2. Enchytraeid reproduction tests: standard and extension

The validity criteria were fulfilled as within the standard OECD test (OECD 220, 2016), i.e., in controls, adult mortality was <20 % and the number of juveniles >50 per replicate, with a coefficient of variation <50 %.

The standard test (28 days) showed that Karate Zeon and lambda-cyhalothrin caused a dose-dependent decrease in *E. crypticus'* survival and reproduction (Fig. 3A). Karate Zeon was slightly more toxic in terms of survival, considering the effects observed at 10 mg/kg. Further there was a tendency for decrease in adults' size (Fig. 3B).

The estimated ECx (Table 1) are similar (and with overlapping confidence intervals) (measurements not possible at 32 mg/kg due to mortality) for both materials.

The effects observed for the standard extension (56 days) are in line with reproduction effects observed at day 28 (Fig. 3C). At the lower tested concentrations (0.32 and 1 mg/kg) Karate Zeon caused an increase in total population, clearly visible from the results over time (Fig. 3D). The size of the adults, measured at day 28 was not significantly affected up to 10 mg/kg (Fig. 3B).

3.2.3. Full life cycle tests

Karate Zeon and lambda-cyhalothrin caused a dose-response reduction in terms of hatching (13d), survival and reproduction (46d) (Fig. 4 A, C). In terms of hatching, significant effects were observed from 10 mg/kg of both materials. Karate Zeon significantly reduced survival and reproduction from 3 mg/kg, while for lambda-cyhalothrin, significant effects on survival occurred from 0.32 mg/kg. Size was not significantly affected by Karate Zeon exposure (Fig. 4B, D). Lambda-cyhalothrin reduced the size of the hatched juveniles (13d), this being significant at 10 mg/kg, and there was a tendency for decrease in size of adults (46d) (measurements not possible at 32 mg/kg due to mortality).

4. Discussion

Karate Zeon is a pesticide formulation, labelled as capsule suspension (CS), but without disclosing the size of the capsules. Its characterization confirmed the presence of micro-capsules and of nano-capsules, up to 100 nm. This is in agreement with the literature data, were a polydisperse sized suspension, with elements in the nanometer size range has been described for Karate Zeon (Meredith et al., 2016; Paz-Trejo et al., 2022; Slattery et al., 2019).

Karate Zeon and lambda-cyhalothrin induced virtually no avoidance behaviour within the tested concentrations. Although the model was applied, and the EC numbers are obtained, this should not be used without consideration - as we know from previous experience, an increase in the concentration will not necessarily correspond to an increase in the endpoint avoidance behaviour when this is impaired by e.g., neurotoxicity. Lambda-cyhalothrin is a pyrethroid insecticide, acting via neurotoxicity as caused by neuronal hyperexcitation, which results in repetitive synaptic firing and persistent depolarization. The molecular targets of pyrethroids are similar in mammals and insects, and include voltage-gated sodium, chloride, and calcium channels, gamma-aminobutyric acid (GABA)-gated chloride channels, nicotinic acetylcholine (Ach) receptors, and intercellular gap junctions (Gupta and Crissman, 2013). It has been shown for *E. crypticus*, that non-avoidance to boric acid (there was in fact an attraction to the toxicant) was associated to interference with the GABAergic system of the animals (Bicho et al., 2015a). Considering the similar modes of action of lambda-cyhalothrin, an interference with any neurotransmission processes is a likely event here, hence with neurotoxic effects (e.g., tremors or paralysis) and impair the avoidance behaviour of *E. crypticus*. This is in agreement with the effects reported in zebrafish embryos exposed to Karate Zeon and lambda-cyhalothrin, which exhibited dose-dependent increase in the number of embryos experiencing tremors up 80 μg lambda-cyhalothrin/L; above 400 μg/L the tremors decreased because there was an increase in the number of paralysed embryos (Meredith et al., 2016). Nevertheless, the earthworms *Eisenia fetida* showed a strong avoidance behaviour to lambda-cyhalothrin (EC50 = 0.3 and 3.3 mg/kg in LUFA 2.2 and OECD soils, respectively) (Garcia et al., 2008), hence there is an obvious species specific response. Earthworms (*Eisenia andrei*) also avoided cypermethrin, another pyrethroid insecticide (Sousa and Andréa, 2011). There is no detailed information on potential difference in neurotransmission processes between earthworms and enchytraeids, but boric acid is an example where this also occurs, being even the reference substance for the earthworms' avoidance test (ISO 17512-1, 2008). This is not the first-time differences are observed between species of the same group, although we must use these as surrogates and group representatives; *E. crypticus* seems to be more sensitive for neurotoxic compounds than *E. andrei/ E. fetida*. The fact that similar toxic responses were observed for Karate Zeon and lambda-cyhalothrin exposures indicates that the common active substance in both materials had a dominant role in toxicity. These nanopesticide capsules were designed to disperse the active substance in two stages: the disruption of thin-walled capsules should lead to a rapid initial release, followed by a slow release provided by thick-walled capsules (Slattery et al., 2019). The rates of release were not determined but it is possible that enough active substance was released within the first 48 h (the duration of the avoidance test) and elicit (neuro)toxicity. Although no avoidance was observed within 48 h (which, as discussed can be a sign of neurotoxicity), when exposure lasts longer (13/46 days from the FLC tests or 28/56 days from the OECD standard and its extension) survival and reproduction were reduced. Hence, in a realistic field exposure scenario, this incapacity to avoid Karate Zeon or lambda-cyhalothrin will pose high hazard to enchytraeids' with its population decline.

Based on the standard OECD test, Karate Zeon toxicity to *E. crypticus* was similar to the toxicity induced by lambda-cyhalothrin. Survival was a less

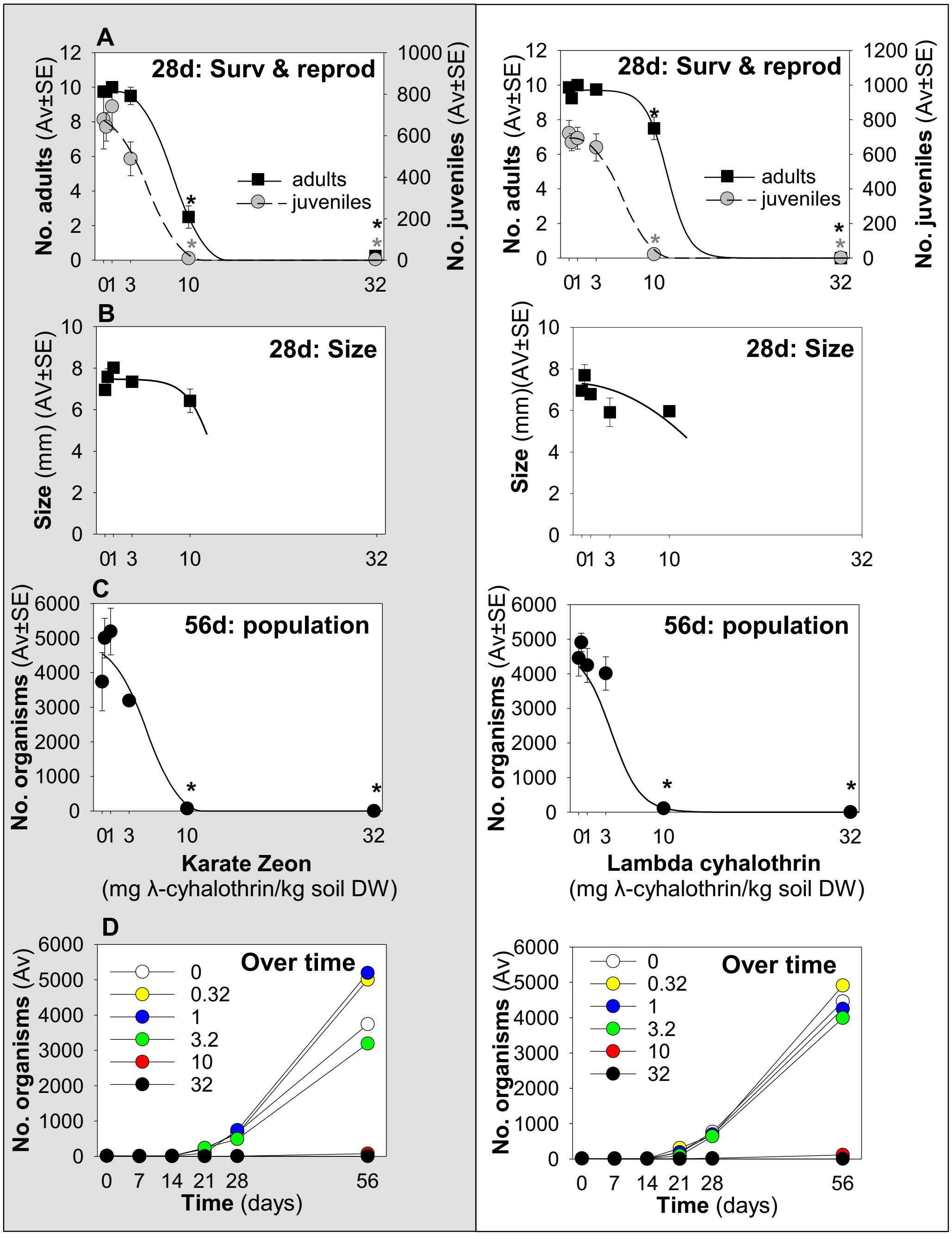

Fig. 3. Results in terms of survival, reproduction, and size from the Enchytraeid Reproduction Test, when exposing *Enchytraeus crypticus,* in LUFA 2.2 soil, to Karate Zeon and its active substance lambda-cyhalothrin for (A, B) 28 days (OECD Standard), (C) 56 days (OECD standard extension), and (D) overview of the time series sampling at days: 7, 14, 21, 28 and 56 days. A-C: Values represent number of adults, juveniles, size, and population as average ± standard error (Av ± SE); D: Values represent the population as average (Av). *: $p < 0.05$ (Dunnett's).

sensitive endpoint than reproduction, this is commonly reported in the literature for several classes of chemicals, including lambda-cyhalothrin in earthworms (Garcia et al., 2011). The lambda-cyhalothrin ECx in *E. crypticus* are lower than those reported for *E. fetida* (LC/EC50 = 11.6/6.3 mg/kg for *E. crypticus* (present data), and LC/EC50 = 140/44.5 mg/kg for *E. fetida* (Garcia et al., 2011)), but in the same range as reported for *E. crypticus* exposed to another pyrethroid insecticide, alpha-cypermethrin (EC50 = 4.91 mg/kg), in a natural soil collected in Norway (Hartnik et al., 2008). Hartnik et al. (2008) results also showed that *E. crypticus* was more sensitive than *E. fetida* to alpha-cypermethrin exposure.

Previous studies showed differences in toxicity between the nanopesticide Kocide 3000 and its active substance and/or non-nanoformulations, to soil invertebrates, either higher toxicity for the nano (Neves et al., 2019) or the opposite (Morgado et al., 2022). To *E. crypticus*, a nanoformulation of atrazine (a herbicide) was less toxic than the active substance atrazine alone (Gomes et al., 2019), and the differences could be related to differentiated mechanisms of uptake and/or cellular transport between the nano and the free form of atrazine (Gomes et al., 2022). Further, for prolonged exposure (56 days), the toxicity patterns in terms of reproduction were maintained, for both Karate Zeon and lambda-cyhalothrin. One study with different forms of the pyrethroid

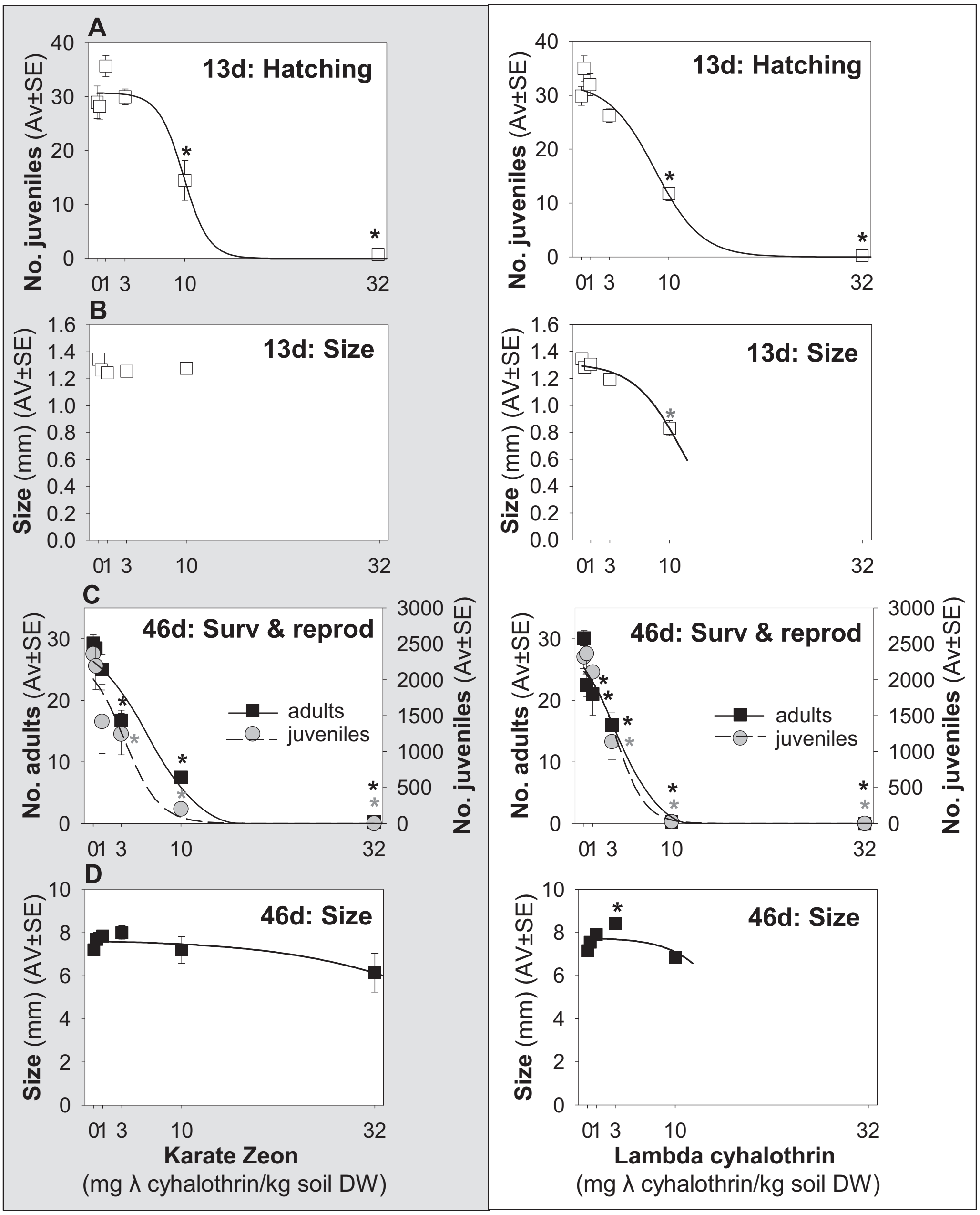

Fig. 4. Results in terms of hatching (13d), survival and reproduction (46d), and size (13 and 46d) from the Full Life Cycle test, when exposing *Enchytraeus crypticus,* in LUFA 2.2 soil, to Karate Zeon and its active substance lambda-cyhalothrin for (A, B) 13 days, and (C, D) 46 days. Values represent number of adults, juveniles, and size as average ± standard error (Av ± SE). *: $p < 0.05$ (Dunnett's).

insecticide bifenthrin: the free active substance, a commercial non-nano formulation and two commercial nanoformulations (the commercial names were not provided, only the producer, Vive Crop Protection), have shown that the half-lives of bifenthrin in soil were higher for the nanoformulations (Mohd Firdaus et al., 2018). Differences were found also in terms of uptake/elimination in earthworms, with higher rates for the bifenthrin nanoformulations. The higher uptake rates lead to higher accumulation of bifenthrin for the nano treatments, but while for the nanoformulations most of the bifenthrin was found in the earthworms' gut, for the active substance. and non-nano formulation, bifenthrin accumulated mostly in the other (other than the gut) tissues (Mohd Firdaus et al., 2018). This study showed that the nanoencapsulation of bifenthrin affected its behaviour and uptake in soil in the following way: decreased sorption to soil, increased persistency, and altered the uptake and distribution in earthworms (Mohd Firdaus et al., 2018). Although we found minor differences between Karate Zeon and lambda-cyhalothrin toxicity to *E. crypticus*, different patterns of uptake and elimination cannot be excluded.

Hatching was also affected similarly by both Karate Zeon and lambda-cyhalothrin, being in the same order of magnitude as the effects observed in terms of 28 days survival (similar ECx), indicating that embryos and adults were equally sensitive to both materials. Also, these results show that for Karate Zeon, cocoons/embryos were actually exposed given the induced toxicity. Thus, we assume that within the 13 days exposure that lasts the hatching test, the nanocapsules must have released enough active substance to produce similar toxicity to the active substance alone. We cannot confirm that the active substance was delivered inside the cocoon membrane, as there is no information available on the cocoon membrane pore-size (Bicho et al., 2021); it could also be that it is delivered outside the membrane to the newly hatched juveniles. One study on synthesized micro- and nanocapsules containing lambda-cyhalothrin showed that the total release of the active substance from the capsules, in water, ranged from 24 h for the nanosized capsules (210 nm) to >8 days for the micro-sized ones (12.4 μm) (Huang et al., 2022). There is no equivalent information for Karate Zeon, but it is a relevant comparison and likely similar circumstance.

Although the effects on hatching did not reveal an increased sensitivity of the embryos stage, the animals exposed from cocoons, for 46 days in the FLCt, were more sensitive than the embryos (13d_EC50 > 46d_LC/EC50) and more sensitive than the animals exposed from the adult stage (ERT test). At this point, lambda-cyhalothrin was slightly more toxic than Karate Zeon, in terms of adults' survival, while the effects on reproduction were similar between the two materials. This increased sensitivity seems to indicate that the juvenile stage is more sensitive, i.e., the growth phase compared to the adult stage. The differences do not seem likely due to the longer exposure duration, as no increased toxicity was observed from 28 to 56 days in the ERT extension. No impact was found in terms of size (day 46) thus, it could be that the exposed animals allocated more energy to growth than to detoxification of lambda-cyhalothrin (both as free or nanoencapsulated forms). Lower investment in detoxification can lead to genotoxicity by the accumulation of damage to the DNA, as reported for Karate Zeon exposed lymphocytes from human peripheral blood (Paz-Trejo et al., 2022).

Nanopesticides are often found to be more effective against target-organisms than non-nano pesticides (Wang et al., 2022), which should lead to lower application rates. Hence, similar toxicity results between Karate Zeon and lambda-cyhalothrin, given in lower application rates, the nanoformulation could still theoretically pose lower risks to non-target species like the enchytraeid population. However, literature data also show that nanoencapsulation increased the soil persistency of the active substance bifenthrin and alters the uptake and distribution in earthworms, highlighting that the risk of an encapsulated pesticide was different, i.e., not predictable from the non-nano product (Mohd Firdaus et al., 2018). Overall, risks assessment of nanoformulations should not be based by default on the risks of its active substances alone.

5. Conclusions

This is the first study reporting the effects of the commercial nanopesticide Karate Zeon to a non-target soil living invertebrate. Karate Zeon was equally toxic to its active substance lambda-cyhalothrin in *E. crypticus* and there was no indication that toxicity would increase in prolonged exposure (based on the 56d results). FLCt results indicated that juvenile life stage was the most sensitive life stage to both Karate Zeon and lambda-cyhalothrin exposure, resulting in an increased toxicity for the survival of adult animals that were exposed from the cocoon stage (as opposed to the exposure during adult stage alone). This is an important observation, that shows impact at the developmental level. The similar toxicity results between Karate Zeon and lambda-cyhalothrin show that, if given at a lower application rate, the nanoformulation could still theoretically pose lower risks to the enchytraeid population and hence it would represent an improved benefit to substitute to an advanced material.

CRediT authorship contribution statement

Susana I.L. Gomes: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Sekerani B. Chidiamassamba:** Methodology, Formal analysis, Writing – review & editing. **Janeck J. Scott-Fordsmand:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Mónica J.B. Amorim:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164664.

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