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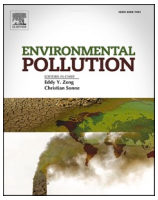

Nanoemulsion carriers for drug delivery: Assessment of environmental hazards☆

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ABSTRACT

Nanoemulsions (NEs) have been extensively studied as carriers for drug delivery, since these provide a good alternative to the existing non-nano systems, while promoting their target delivery and controlled release. NEs are considered safe drug carriers from a pre-clinical perspective, but there is currently no information on their ecotoxicological effects. In the present study we investigated the toxicity of a NE material (lecithin, sunflower oil, borate buffer) designed to be used as a liposomal excipient for eye drops, further referred to as (Lipid Particle:LP) LP_Eye and its dispersant (borate buffer) (LP_Eye disp.). Effects were assessed using two model species in soil ecotoxicology in LUFA 2.2 soil: *Enchytraeus crypticus* (Oligochaeta) and *Folsomia candida* (Collembola), based on the OECD standard guideline (28 days) and its extension, a longer-term exposure (56 days). The endpoints evaluated included survival, reproduction, and size. LP_Eye and LP_Eye disp. were toxic to *E. crypticus* and *F. candida*, affecting all measured endpoints. The toxicity of LP_Eye in *E. crypticus* seemed to be induced by the dispersant, whereas for *F. candida*, more sensitive, this was less explanatory. There were no indications that toxicity increased with longer exposure. Current results provide ecotoxicological data for a group of NMs that was absent, revealing toxicity to relevant environmental species. Indications were that the dispersant contributed to most of the observed effects, thus there is room to improve the formulation and achieve lower environmental impact.

1. Introduction

Lipid-based nanomaterials, such as nanoemulsions (NEs: liquid-in-liquid dispersions with droplet sizes in the nanometer range) received a lot of interest from researchers, with many possible applications, e.g., pharmaceutical, cosmetics and food (Azmi et al., 2019). Whitin the pharmaceutical industry, NEs have been proposed as drug delivery systems because of their capacity to solubilize non-polar active compounds (Azmi et al., 2019) associated to controlled substance release and specific targeting (Khiev et al., 2021). Research on NEs with therapeutical applications include, among others, anticancer therapies (e.g. (Ganta et al., 2014b, 2014a; Primo et al., 2008)) and antifungal drug (e.g. (Hussain et al., 2016)). However, it is in the field of ophthalmic formulations that NEs emerged as the most promising solution to improve the delivery of ophthalmic drugs (Gawin-Mikołajewicz et al., 2021). This is because lipid nanoparticles (LP) address very specific limitations such as overcoming the corneal barriers and increasing corneal retention (Battaglia et al., 2016; Fernandes et al., 2021a; Gawin-Mikołajewicz et al., 2021; Khiev et al., 2021). Despite the obvious potential, any new product must undergo not only clinical, but also environmental safety assessment prior mass production and commercialization.

Toxicity studies on NEs are scarce. Among the few examples, it was shown that a thymoquinone (bioactive compound present in the black seeds of the *Nigella sativa* plant) - rich fraction NE was not toxic to Sprague Dawley rats, administered orally at 20 mL/kg, considering the parameters general behaviour, body weight, food and water consumption, relative organ weight, haematology, histopathology, and clinical biochemistry (Tubesha et al., 2013).

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Considering NEs for ocular use in particular, most of the studies focus on testing the developed product in terms of eye irritation, i.e. considering mostly the product application. For example, for moxifloxacin (antibiotics) NE, Shah et al. (2019) tested not only albino rabbits eye irritation, without significant effects, but also, the antimicrobial activity of the NE against the target pathogens. Results showed as good or better antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in comparison to the commercial (non-nano) eye drops of moxifloxacin (Shah et al., 2019). The selection of the best NE (the best long-term stable nanoemulsion for the ocular administration of triamcinolone acetonide) was based on cytotoxicity and genotoxicity studies on ARPE-19 and HMC3 cell lines (arising retinal pigment epithelia and microglial cell lines, respectively) (Fernandes et al., 2021b), without further testing on non-target cells or organisms. A NE with ophthalmological applications (the same tested in the present study) was not cytotoxic to fish cell lines within 24h (IC50 $<$ 100 μL/mL), however long-term cytotoxicity was observed, as after 10 days of exposure, 100 μL/mL of the NE reduced RTgill-W1 cells viability in 50% (IT50) (Hernández-Moreno et al., 2022). Hence, besides this in vitro study, there is currently no information on the ecotoxicity of lipid-NPs with ocular application, being a major gap for the environmental safety of those materials. The scenario for NEs, in general, is not very different. To the best of our knowledge, the only study on the ecotoxicity in soil invertebrates - the collembolan *Folsomia candida* - to a NE (containing cinnamon oil) showed no toxicity up to 100 mg oil/kg soil (Volpato et al., 2016).

The assessment of the environmental impacts of medicinal products, as within the European Medicines Agency (EMA), is mandatory and must be performed during the development of new medicines. As highlighted by Amorim et al. (2020), although no legal definition of nanomedicine or nanobiomaterial exists, their use as medicines states their coverage within EMA Directive 2001/83/EC, Article 8 (3). Hence, it is key to assess the environmental hazards materials like NEs with relevant applications. The full formulation components, e.g. its preservatives (Coroi et al., 2015) have a function and can have a toxic impact, thus this should be considered.

In the present study, we investigated the environmental effects of a liposomal excipient for eye drops, further referred to as LP_Eye, and its dispersant (further referred to as LP_Eye disp.). Two soil model species *Enchytraeus crypticus* (Oligochaeta) (OECD 220, 2016) and *Folsomia candida* (Collembola) (OECD 232, 2016) were selected as test species to cover different routes of exposure and life traits in soil. Effects were assessed based on the OECD standard (28 days) reproduction tests (OECD 220, 2016; OECD 232, 2016) and the standard extension, a longer-term exposure (56 days) (Guimarães et al., 2019a; Ribeiro et al., 2018). The experimental design cover long (er)-term effects: survival and reproduction, as recommended for nanomaterials, but also adds the endpoints survival at intermediate times (7, 14, 21 days) and size measurements (at 28 days for *E. crypticus* and at 28 and 56 days for *F. candida*).

The aim of this study was to assess the toxicity of a nanoemulsion in the terrestrial ecosystem, a mandatory requirement. This will provide ecotoxicological data for a group of NMs for which such information is extremely limited, filling an important knowledge gap.

2. Materials and methods

2.1. Test organisms

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) were cultured under controlled conditions of temperature (20 ± 2 °C) and photoperiod (16:8 h light:dark) in agar, consisting of sterilized Bacti-Agar medium (Oxoid, Agar No. 1, Thermo Scientific, Waltham, MA, USA) and a mixture of four different salt solutions at the final concentrations of 2 mM $CaCl_2{\cdot}2H_2O$ (ACS reagent, ≥99%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 1 mM $MgSO_4.7H_2O$ (ACS reagent, ≥98%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.08 mM KCl (ACS reagent, 99.0–100.5%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 0.75 mM $NaHCO_2$ (ACS reagent, ≥99.7%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The organisms were fed with ground autoclaved oats twice per week. Cultures were synchronized to obtain 18–20 days old organisms (for further details on culture synchronization see Bicho et al. (2015a)).

Folsomia candida (Collembola) were cultured on a moist substrate of plaster of Paris (powder, Grouht Soluções Químicas Lda., Barcelos, Portugal) and activated charcoal (powder, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (8:1 ratio), at 20 ± 2 °C, under a photoperiod of 16:8 (light:dark). The organisms were fed once a week with dried baker's yeast (*Saccharomyces cerevisiae*). Cultures were synchronized to obtain synchronized age juveniles (10–12 days old).

2.2. Test soil

The natural standard LUFA 2.2 soil (LUFA Speyer, Germany) was used for the experiments and is characterized as follows: pH (0.01M $CaCl_2$): 5.6 ± 0.4; organic carbon: 1.71 ± 0.30%; cation exchange capacity (CEC): 9.2 ± 1.4 meq/100g; maximum water holding capacity (maxWHC): 44.8 ± 2.9 g/100g; texture: 8.0 ± 1.5% clay, 13.7 ± 1.0% silt, and 78.3 ± 1.0% sand content.

2.3. Test materials

LP_Eye is a colloidal water suspension of nanodrops of lecithins and sunflower oil, having an intended use as liposomal excipient for eye drops, registered as medical device, with indication for dry eye. The particles were obtained by warm microemulsion method. Briefly, the following components were mixed in a turbo emulsifier equipment: water (borate buffer pH 7.4), soy lecithin (Merck, Darmstadt), sunflower oil (USP, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), ascorbyl palmitate and alpha tocopheryl acetate ((Merck, Darmstadt) were added as antioxidant agents. After vigorous stirring an homogeneous liposomal dispersion was obtained, with high lipid concentration, which was then diluted in borate buffer to final concentration. The final lipid content (lecithin and oil) is 7.5 mg/mL (0.75% w/v). The dispersion was then processed for final sterilizing filtration at 0.22 μm and directly stored in sterile plastic bags for shipment (Flexboy®, 10 L, Sartorius, Göttingen, Germany). The test materials include the LP_Eye, and its dispersant (46 mM borate buffer, pH 7.4). A summary of LP_Eye characteristics can be found in Table 1.

2.4. Materials characterization

LP_Eye was characterized by Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and electrophoretic light scattering (ELS). TEM was performed by using a JEOL-JEM 1010 microscope

Table 1
Summary of LP_Eye characterisation, including the composition (ingredients), hydrodynamic size (DLS) and zeta potential (ELS); LP_Eye were manufactured with the dispersant (disp.) borate buffer pH 7.4 – LP_Eye disp. DLS: dynamic light scattering; PDI: polydispersity index.

Material	LP_Eye	LP_Eye disp.	Technique
Complete formulation	water (borate buffer pH 7.4), soy lecithin, sunflower oil (USP), ascorbyl palmitate, alpha tocopheryl acetate	water (borate buffer pH 7.4)	–
Z-average (nm)/PDI	153.2/0.193	–	DLS
Z-potential (mV)	−47.0	–	ELS
Osmolarity (mOsmol/kg)	188.0	–	OSMOMAT

(JEOL USA inc., Peabody, MA, USA) operating at an acceleration voltage of 100 kV. TEM images were acquired to measure size and characterize nanomaterial morphology. The sample was diluted 1:100 in MilliQ water and placed onto a carbon-coated grid and dried at room temperature under vacuum. DLS measurements were carried out with a Zeta-Sizer Malvern Instrument (Malvern Panalytical, Alfatest, Rome, Italy) in backscattering mode. All studies were performed at a 173° scattering angle with temperature controlled at 25 °C in 1 mL polystyrene cuvettes. Further, characterization in terms of size and Zeta-potential (ELS) were performed in auto-mode at 25 °C, for a total of 15 min with 3 consecutive measurements for each sample.

2.5. Spiking procedures

The tested concentrations were 0, 50, 100, 150, 200 and 1000 mg LP_Eye/kg soil dry weight, and 0, 100, 150, 200 and 1000 mg LP_Eye disp./kg soil dry weight. Spiking followed the recommendations for nanomaterials (OECD, 2012) as aqueous dispersions onto the pre-moistened soil. Stock aqueous dispersion was serially diluted, and spiking was done per individual replicate to ensure total raw amounts of the tested material. In short, 20/40 g (for *E. crypticus*) or 30 g (for *F. candida*) of pre-moistened soil per replicate was thoroughly mixed with the corresponding amount of the test materials to obtain the final concentration range. Deionised water was added to achieve 50% of soil maxWHC and the soil was homogeneously mixed again. Soil was left to equilibrate for 1day prior test start.

2.6. Test procedures

2.6.1. Enchytraeus crypticus

Tests with enchytraeids followed the standard guideline (OECD 220, 2016) (28 days), plus the OECD extension (56 days), as described in e.g. Ribeiro et al. (2018). In short, the standard test was extended 28 more days (56 days in total) and extra monitoring sampling times were added at days 7, 14, 21, (28) and 56 days. Endpoints included survival for all sampling days, reproduction at days 28 and 56, i.e. number of juveniles and population, respectively, and size at day 28, to assess impact on growth. Four replicates per treatment were done, except at days 7, 14 and 21, with one replicate. Ten synchronized age organisms (18–20 days old after cocoon laying) were introduced in each test vessel with moist soil (⌀4 cm with 20 g of soil for exposure up to day 28, and ⌀5.5 cm with 40 g of soil for exposure up to day 56) and food supply (22 ± 2 mg, autoclaved rolled oats). Test ran up to 56 days at 20 ± 1 °C and 16:8h photoperiod. Food (11 ± 1 mg: until day 28, and 33 ± 3 mg: from 28 to 56 days) and water were 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 fixated for 24 h with ethanol (96% vol, AGA S.A., Prior Velho, Portugal) and Bengal rose (Dye content 95%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (1% in ethanol), 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, and size (length) was assessed using the software ImageJ (v.1.52a, Wayne Rasband, National Institutes of Health, USA).

2.6.2. Folsomia candida

Tests with collembolans followed the standard guideline (OECD 232, 2016) (28 days) plus the OECD extension (56 days), as described in Guimarães et al. (2019a, 2019b), representing one more generation compared to the standard. In short, the standard test was extended for 28 more days (56 days in total), and extra monitoring sampling times were done at days 7, 14, 21, (28) and 56 days. At all sampling points, endpoints included survival and reproduction, i.e., number of juveniles (or population at day 56). Size of organisms was assessed at days 28 and 56, to assess impact on growth. Four replicates per treatment were done, except at days 7, 14 and 21, with one replicate. Ten synchronized age animals (10–12 days old) were placed in each test vessel with moist soil (⌀5.5 cm, 30 g of soil) and food supply (2–10 mg, baker's yeast). Test ran up to 56 days at 20 ± 1 °C, under a 16:8h photoperiod. Food and water were replenished every week. At each sampling day (7, 14, 21, 28 and 56 days), the test vessels were flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further analyses (count and measure (size, area)) using the software ImageJ (v.1.52a, Wayne Rasband, National Institutes of Health, USA). For the replicates that continued until day 56, after the similar flooding and photographing procedure, the sampled juveniles at day 28 were transferred with a spoon to a box with a layer of Plaster of Paris to absorb extra water from the spoon. After this, ten of the biggest juveniles (ca. 11 days old) were transferred to new test vessels containing soil (spiked at day 0), representing an F1 exposure and the test ran under the same exact conditions as F0. At day 56, survival (F1) and reproduction (F2) were counted and measured, following the previously described procedure.

2.7. Data analysis

One-way analysis of variance (ANOVA), followed by the Dunnett's Post-Hoc test was used to assess differences between control and treatments for all endpoints (survival, reproduction, and size) (SigmaPlot, SPSS Statistics for Windows, version 14.0 (SPSS Inc., Chicago, Ill., USA)). Effect concentrations (ECx) were calculated modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program software (TRAP v1.30a, USEPA).

3. Results

3.1. Materials characterization

TEM images (Fig. 1) showed that the lipid particles are polydisperse

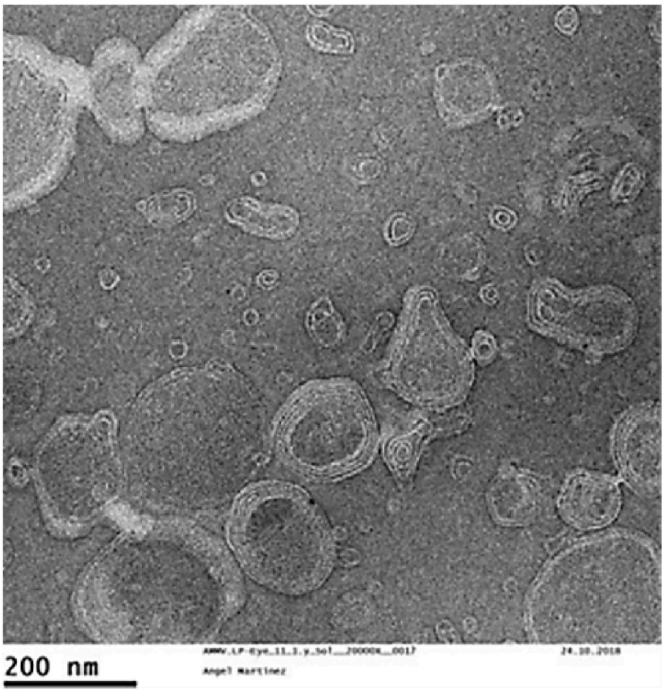

Fig. 1. Transmission Electron Microscopy (TEM) image of LP_Eye nanoemulsion.

in size, with diameters ranging from 50 to 400 nm.

Size range observed in TEM images are in line with DLS results, which showed a mean hydrodynamic diameter of 153 nm, with 47.5% of polydispersity calculated by cumulant analysis. The Zeta-potential is negative, −47 mV, and consistent with its fatty acid composition.

3.2. Ecotoxicological tests

The validity criteria were fulfilled for the *E. crypticus* tests, 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%.

LP_Eye and the LP_Eye dispersant caused a dose-dependent reduction in survival and reproduction of *E. crypticus* (Fig. 2).

Reproduction was more affected than survival, with significant reduction from 100 mg/kg, for both LP_Eye and LP_Eye disp., while survival was not affected up to 200 mg/kg. The effects observed after prolonged exposure (56 days) resemble the effects on day 28 reproduction, and with similar ECx values (Table 2). The size of the animals exposed, for 28 days showed a decrease at 200 mg/kg, significant for LP_Eye, although there was an increase up to 100 mg/kg (LP_Eye and LP_Eye disp.) (Fig. 3).

F. candida survival was inhibited by LP_Eye from 100 mg/kg, and by LP_Eye dispersant from 150 mg/kg, in a dose-dependent way (Fig. 4A). Reproduction was almost null in all tested concentrations, for both LP_Eye and LP_Eye disp. (100% effect at 150 mg/kg).

The size of the surviving animals (up to 100 mg/kg) was significantly reduced in comparison to control (Fig. 3B). There were not enough juveniles at day 28 to continue the exposure for the second generation, thus graphs and ECx values (Table 1) are shown for 28 days exposure only.

4. Discussion

F. candida was more sensitive than *E. crypticus* to LP_Eye exposure (LC50 = 96 and 289 mg LP_Eye/kg soil, for *F. candida* and *E. crypticus*, respectively). The toxicity patterns of LP_Eye and LP_Eye disp., in terms of reproduction for *E. crypticus*, were maintained after 56 days of exposure, while *F. candida* did not produce enough juveniles for a second-generation exposure.

Reproduction was more sensitive than survival, as often reported in the literature for many chemicals, including (but not limited to) nanomaterials: copper (Cu) (Gomes et al., 2015a), nickel (Ni) (Santos et al., 2017), silver (Ag) (Mendes et al., 2015; Rodrigues et al., 2020), and for instance a pesticide nanoformulation (Gomes et al., 2019).

In *E. crypticus*, the effects caused by LP_Eye were like those induced by LP_Eye dispersant (reproduction EC50 = 75 and 88 mg/kg, for LP_Eye and LP_Eye disp., respectively), suggesting that the dispersant composition of the formulation (borate), and not the lipidic ingredients, are causing the observed toxicity. This is in agreement with results from Le Roux et al. (2017), where lipid nanocapsules (empty from any drug) of 25, 55 and 100 nm caused toxicity to RAW264.7 cells, due to one of the surfactants present in the formulation. Among the ingredients of LP_Eye formulation is the borate buffer. Boric acid (used to produce borate buffer) is known to reduce enchytraeids and collembolans survival and reproduction, and without effects on their avoidance behaviour (Amorim et al., 2012). In the case of *E. crypticus*, the non-avoidance of boric acid was associated with neurotoxic effects via the Gamma-Aminobutyric Acid (GABA)ergic system mechanism, where boric acid acts as a $GABA_A$ receptor antagonist causing anaesthetic effects (Bicho et al., 2015b). The ECx reported in the literature for enchytraeids [*Enchytraeus albidus* LC/EC50 = 325/104 mg/kg in LUFA 2.2 soil (Amorim et al., 2012); *E. crypticus* EC50 = 220 mg/kg in OECD soil (Becker et al., 2011)] and for collembolans [*F. candida* LC/EC50 = 139/54 mg/kg in LUFA 2.2 soil (Amorim et al., 2012)] are close to the ECx determined here, indicating that the dispersant borate buffer must contribute for the toxicity of LP_Eye. As observed for LP_Eye, boric acid was more toxic to collembolans than to enchytraeids (Amorim et al., 2012), further supporting the results and in agreement with the species sensitivity distribution. Nevertheless, the amount of boric acid present in the LP_Eye is about 30% of the total formulation, thus not fully explaining the toxicity observed.

For *F. candida*, survival was more affected by LP_Eye than LP_Eye disp., hence in this case suggesting that toxicity was not only caused by the dispersant, and that the lipid portion of the formulation must contribute for the observed effects. No differences between LP_Eye and LP_Eye disp. were observed in terms of reproduction, probably due to the high toxicity concentration range. Vegetable oils have been

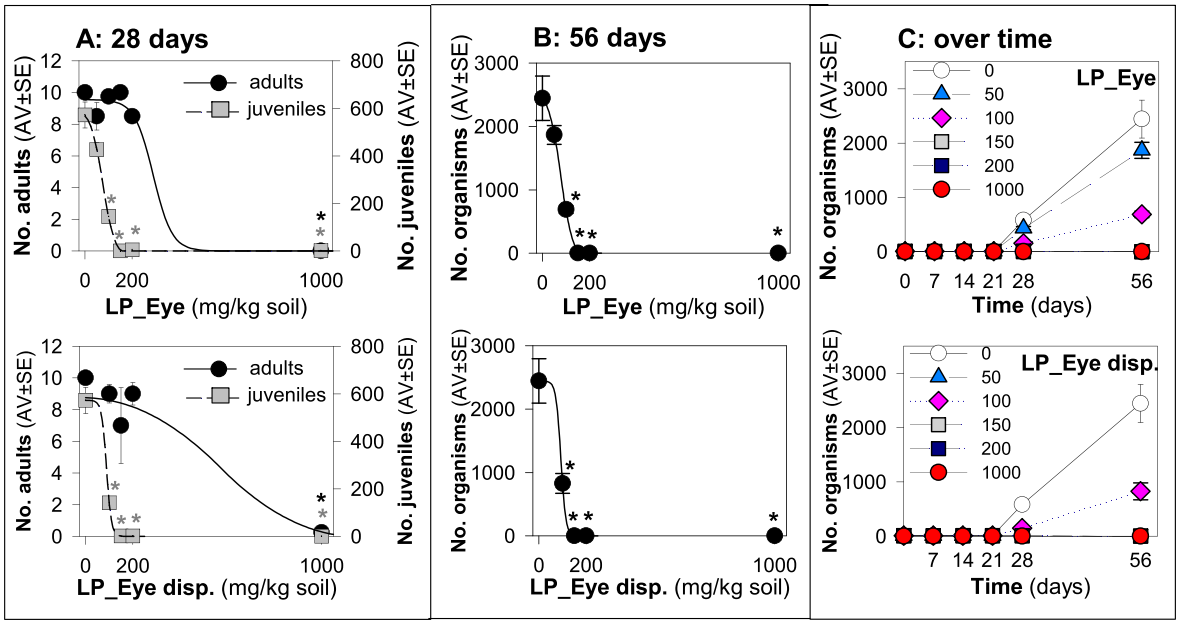

Fig. 2. Results in terms of survival and reproduction when exposing *Enchytraeus crypticus* in LUFA 2.2 soil to LP_Eye and LP-Eye disp., during (A) 28 days (OECD Standard), (B) 56 days (OECD standard extension), and (C) overview of the time series sampling at days: 7, 14, 21, 28 and 56. Values represent number of adults, juveniles, and population as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's).

Table 2
Summary of the effect concentrations (ECx with 95% confidence intervals – CI), expressed as mg LP_Eye or LP_Eye disp. per kg soil (dry weight), for *Enchytraeus crypticus* and *Folsomia candida* exposed LP_Eye and LP_Eye disp., in LUFA 2.2 soil. The models used are Logistic 2 parameters (Log2P) or Threshold sigmoid 2 parameters (Thres2P). S: slope; Y0: top point; n.d.: not determined.

Test material/species	Endpoint	Time (days)	EC10 (95% CI)	EC50 (95% CI)	EC90 (95% CI)	Model & Parameters
LP_Eye						
E. crypticus	Survival	28	202 (155–249)	289 (53–525)	376 (-71-823)	Log 2P; S: 0.006, Y0:9.6, r^2:0.9
	Reprod	28	28 (12–43)	75 (67–82)	104 (89–120)	ThresSig 2P; S:0.012, Y0:572.5, r^2:0.9
	Total organisms	56	30 (6–54)	78 (66–89)	107 (83–132)	ThresSig 2P; S:0.012, Y0:2444, r^2:0.9
	Size	56	168 (150–187)	219 (202–236)	251 (216–285)	ThresSig 2P; S:0.01, Y0:9.7, r^2:0.9
F. candida	Survival	28	87 (n.d.)	96 (n.d.)	106 (n.d.)	Log 2P; S:0.058, Y0: 9.4, r^2:0.9
	Reprod	28	<50	<50	<50	–
	Size-adults	28	<50	<50	<50	–
	Size-juvs	28	<50	<50	<50	–
LP_Eye dispersant						
E. crypticus	Survival	28	249 (-183-680)	569 (223–915)	767 (-1571-3104)	ThresSig 2P; S: 0.002, Y0:8.7, r^2:0.7
	Reprod	28	66 (-200-331)	88 (1–178)	111 (24–198)	Log 2P; S:0.024, Y0:572.5, r^2:0.9
	Total organisms	56	70 (-134-275)	93 (45–141)	116 (7–225)	Log 2P; S:0.024, Y0:2444, r^2:0.9
	Size	56	221 (n.d.)	182 (n.d.)	151 (n.d.)	ThresSig 2P; S:0.014,Y0:9.7, r^2:0.9
F. candida	Survival	28	99 (94–105)	117 (n.d.)	128 (121–136)	ThresSig 2P; S:0.031, Y0:9, r^2:0.9
	Reprod	28	<100	<100	<100	–
	Size-adults	28	<100	<100	<100	–
	Size-juvs	28	<100	<100	<100	–

For *F. candida* tests, the validity criteria were fulfilled, as within the standard OECD guideline (OECD 232, 2016), i.e., in controls, adults' mortality was <20%, the number of juveniles >100 per replicate, and coefficient of variation <30%.

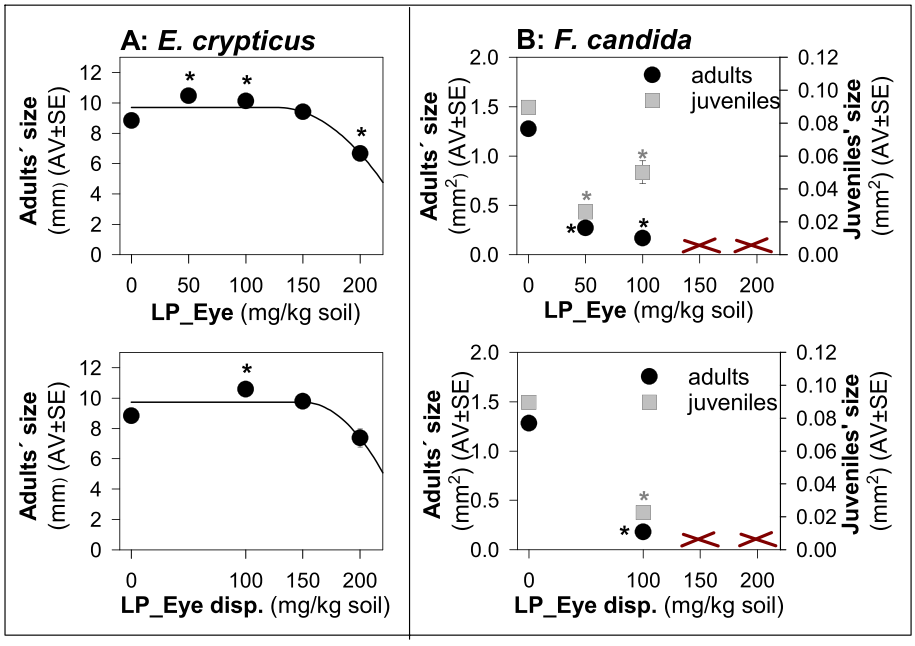

Fig. 3. Results in terms of adults' size when exposing A) *Enchytraeus crypticus* and B) *Folsomia candida*, in LUFA 2.2 soil to LP_Eye and LP-Eye disp., for 28 days. Red crosses indicate absence of measurements due to animals' mortality. Values represent size (length in mm or area in mm^2) as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proposed to remediate polycyclic aromatic hydrocarbons (PAHs) contaminated soils, where the oils facilitate the degradation of the PAHs by soil microorganisms (Thode Filho et al., 2017; Yap et al., 2010). While vegetable oils have been shown to reduce growth in plants (Gong et al., 2008; Hussain et al., 2019), toxicological studies on the possible effects of vegetable oils on soil invertebrates are scarce. *Eisenia fetida* significantly avoided soil containing filtered cooking oil waste above 8% v/w of oil (Thode Filho et al., 2017), but *E. fetida* survival was not

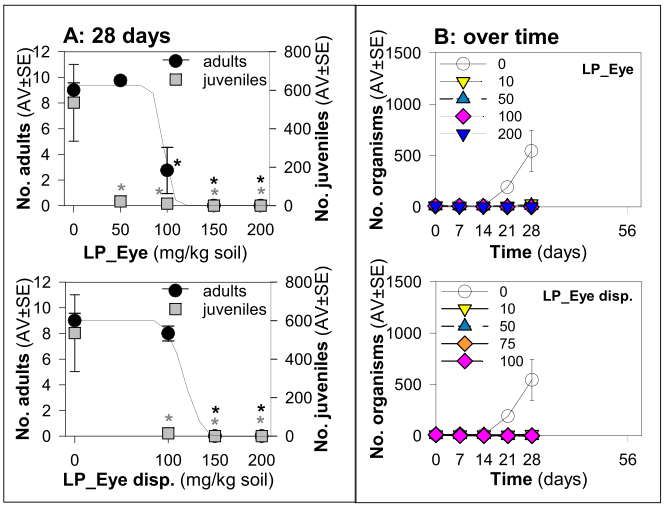

Fig. 4. Results in terms of survival and reproduction when exposing *Folsomia candida* in LUFA 2.2 soil to LP_Eye and LP_Eye disp., during (A) 28 days (OECD Standard), and (B) overview of the time series sampling at days: 7, 14, 21, 28 and 56. Values represent number of adults, juveniles, and population as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's).

affected by 7.5% v/w of soy oil and used soy oil (after frying) up to 180 days of exposure (Tamada et al., 2012). However, an LC50 of 15.6% w/w was also reported for *E. fetida* exposed to soybean oil, associated with oxidative stress and damage to the worms (Du et al., 2023). *F. candida* survival and reproduction was inhibited in soil contaminated (in situ) with olive mill waste (OMWCS) and in soil spiked with olive mill wastewater (OMWW) [LC50/EC50_OMWCS = 45.36/19.44%, LC50/EC50_OMWW = 32.34/10.10%] (Kovačević et al., 2022), but these data are not related to oil alone, thus a direct comparison is not possible. In the current study, we found that the concentration 100 mg LP_Eye/kg soil, which corresponds to 1.33% v/w of oils (lecithin and oil), significantly reduced *E. crypticus* reproduction, and *F. candida's* survival. However, we cannot rule out the toxic role of the dispersant.

The size of the surviving collembolans was significantly reduced, while for enchytraeids an hormesis effect was observed, i.e., size increased at lower concentrations followed by a decrease in higher concentrations. The effects of lipid-based nanomaterials on soil invertebrates are unknown, but soybean oil was shown to reduce the growth rate of *E. fetida* at 5.2% w/w, a concentration that did not affect worms' survival, but completely inhibited its reproduction (Du et al., 2023). For *E. crypticus*, at lower concentrations, LP_Eye (and oils) might have worked as an energy source, as lipids constitute more than 50% of energy reserves in *E. crypticus* (Gomes et al., 2015b). The enchytraeids' investment on growth might have reduced the energy available for reproduction, which was significantly impaired at 100 mg LP_Eye/kg. At 200 mg LP_Eye/kg, enchytraeids are probably investing on detoxification (for example from oxidative stress, as induced by soybean oil in *E. fetida* (Du et al., 2023), or from the dispersant or other ingredients of the formulation) to survive but were not able to reproduce and its size was also reduced.

The differences between species sensitivity could be related to their biology. *F. candida* dwells on the of the topsoil while *E. crypticus* is constantly buried, thus we would expect higher dermal exposure for the enchytraeids. However, collembolans have a unique structure, a ventral tube - collophore, involved in the osmoregulation. Exposure to oily substances can impair their osmoregulation processes, for instance by clogging the collophore. Although enchytraeids exchange air and water through their skin, and thus oil exposure would also be expected to impair their respiration and osmoregulation, the relatively lower effects might be due to surface area differences that offer enchytraeids a compensation - (the surface area of enchytraeids' skin is larger than the collembolans' collophore). Soybean oil exposure did not induce damage to *E. fetida*'s epidermis or other tissues, at a concentration that affects growth and reproduction (Du et al., 2023). No similar study exists for collembolan species, for comparison.

5. Conclusions

The nanoemulsion formulation LP_Eye, designed as liposomal excipient for eye drops, was toxic to both *E. crypticus* and *F. candida* – two soil ecotoxicology model species. LP_Eye impacted survival, reproduction, and size, in a dose-dependent way, although without indication of increased toxicity with prolonged exposure (56 days). *F. candida* was more sensitive than *E. crypticus*, which could be related with their different life traits and physiology. The dispersant alone played a major role in the observed toxicity, although the lipidic-part of the formulation seemed to have also contributed (particularly to *F. candida*).

Credit authors statement

Susana I.L. Gomes: Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Bruno Guimarães**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Paolo Gasco**: Conceptualization, Methodology, Writing – review & editing. **Magda Blosi**: Conceptualization, Methodology, Writing – review & editing. **Ana L. Costa**: Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Janeck J. Scott-Fordsmand**: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Mónica J.B. Amorim**: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

Data availability

Data will be made available on request.

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