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Advanced materials - Food grade melatonin-loaded Lipid Surfactant Submicron Particles (LSSP)–environmental impacts

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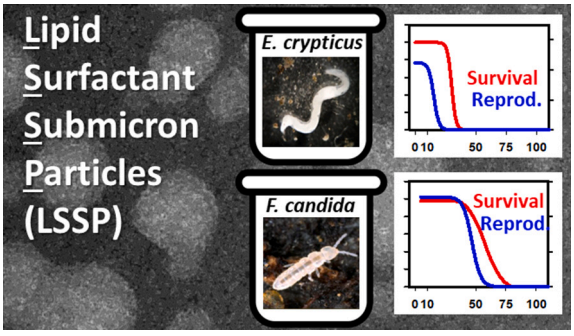
HIGHLIGHTS

- Ecotoxicity of Advanced Materials (AdMa) is poorly known.
- Impacts of Lipid Surfactant Submicron Particles (LSSPs) in *E. crypticus* and *F. candida*.
- LSSPs were toxic to both species: reduced survival and reproduction.
- Prolonged exposure to LSSPs did not increase toxicity.
- Effects on survival and reproduction could not be discriminated between components – typical challenge for AdMa.

GRAPHICAL ABSTRACT



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ABSTRACT

Lipid-based nanoparticles (LNPs) are advanced materials (AdMa), particularly relevant for drug delivery of poorly water-soluble compounds, while also providing protection, stabilization, and controlled release of the drugs/active substances. The toxicological data available often focus on the specific applications of the LNPs-drug tested, with indication of low toxicity. However, the ecotoxicological effects of LNPs are currently unknown. In the present study, we investigated the ecotoxicity of a formulation of Lipid Surfactant Submicron Particles (LSSPs) loaded with melatonin at 1 mg/mL. The LSSPs formulation has been developed to be fully compliant with regulatory for its potential use in the market and all components are food additives. The same formulation without the thickening agent xanthan gum (stabilizer in water phase) designated as LSSP-xg, was also tested. Two soil model invertebrate species were tested in LUFA 2.2 soil: *Enchytraeus crypticus* (Oligochaeta) and *Folsomia candida* (Collembola). Effects were assessed based on the OECD standard guideline (28 days) and its extension, the longer-term exposure (56 days). Assessed endpoints were survival, reproduction, and size. LSSPs and LSSP-xg were toxic to *E. crypticus* and *F. candida* reducing their survival and reproduction in a dose-dependent way: e.g., 28-day exposure: *E. crypticus*: LC/EC50 = 30/15 mg LSSPs/kg soil and *F. candida* LC/EC50 = 55/44 mg LSSPs/kg soil, with similar values for LSSP-xg. Size was also reduced for *F. candida* but was the

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least sensitive endpoint. There were no indications that toxicity increased with longer term exposure. The results provide relevant information on ecotoxicity of a AdMa and highlights the need for awareness of the potential risks, even on products and additives usually used in food or cosmetic industry. Further information on single components and on their specific assembly is necessary for the interpretation of results, as it is not fully clear what causes the toxicity in this specific AdMa. This represents a typical challenge for AdMa hazard assessment scenario.

1. Introduction

Lipid-based Nanoparticles (LNPs) are a wide group of carriers which generically consists of a lipid matrix surrounded by a surfactant layer, having tremendous potential for drug delivery, not only in the pharmaceutical and biomedical industries (Kumar, 2019; Scioli Montoto et al., 2020), but also in the food industry (Fathi et al., 2012; Lu et al., 2021; Nahum and Domb, 2021). The recognized advantages of LNPs as delivery vehicles include the easy loading of drugs or active substances (particularly relevant for poorly water-soluble substances), the good biocompatibility, protecting the incorporated active compounds against degradation, and allowing target delivery and controlled release. These advantages are equally relevant in other fields, e.g. agriculture, applied to pesticides and fertilizers (Grillo et al., 2021; Scott-Fordsmand et al., 2022) or food industry: protection, stabilization and controlled release (Nahum and Domb, 2021). One key aspect for any new material, in particularly those for human use – clinical applications or food additives – relates to its safety, i.e., no toxicity. Naturally, such materials can reach the environment, either by accidental spills during production or use phase, or in the end of life, e.g. as wastes after processing and disposal. Thus, environmental effects should be thoroughly assessed.

Most of the available studies reveal low (cyto)toxicity for several LNPs materials (Fonseca-Gomes et al., 2020), but they often focus on the specific applications of the LNPs tested. For instance, rifabutin (antitubercular drug) loaded LNPs formulations revealed low cytotoxicity to the lung cell lines A549 and Calu-3 (Gaspar et al., 2016). Erythropoietin (a drug prescribed to regulate the red blood cell count) loaded LNPs caused no cytotoxicity to human foreskin fibroblast cell lines Hu02, IBRC C10309, and was more effective than erythropoietin alone on elevating the red blood cell count, haemoglobin, and hematocrit levels in Wistar rats treated by intraperitoneal injection (Dara et al., 2019). Clofazimine loaded LNPs, designed to provide oral drug delivery in leprosy therapy, caused little effects on cell viability and were significantly less cytotoxic to the intestinal model cell lines Caco-2 and HT29-MTX and to the gastric model cell line MKN-28 than the drug clofazimine alone (Chaves et al., 2018). The encapsulation of edelfosine (alkyllysophospholipid antitumor drug with severe side effects) into LNPs, reduced its toxicity to mice, after oral administration (Lasa-Saracíbar et al., 2014). A water-based colloidal suspension of the same Lipid Surfactant Submicron Particles (LSSPs) as tested in the current study, was not cytotoxic or genotoxic to primary HCoEpiC and immortalised Caco-2 and HCT116 epithelial intestinal cells (Antonello et al., 2022). The same suspension was also not toxic to the fish cell line RTgill-W1 after acute 24 h or longer-term 28 days of exposure (Hernández-Moreno et al., 2022). As illustrated by the previously mentioned studies, and as reviewed by Doktorovová et al. (2016), lipid based nanocarriers are overall considered safe. However, most studies have focused on cytotoxicity of lipid-based nanostructures, with limited data on the genotoxic potential of those materials (Azarnezhad et al., 2020). Even though, it is recognized that epigenicity and genotoxicity are two toxicity phenomena which may stimulate cancer progression (Azarnezhad et al., 2020). Currently, the ecotoxicity information for LNPs is very limited. Among the studies performed in soil, one showed that a nanoemulsion containing cinnamon oil was not toxic to *Folsomia candida*, up to 100 mg oil/kg soil (Volpato et al., 2016). However, another study showed that a lipid-based nanosuspension, a colloidal water suspension of nanodrops of lecithin and sunflower oil, inhibit the survival and reproduction of *Enchytraeus crypticus* and *F. candida*, with the reproduction effect concentration EC50 = 75 and <50 mg/kg for *E. crypticus* and *F. candida*, respectively (Gomes et al., 2023b). These results highlight that, at least until the establishment of a larger dataset, the ecotoxicity of LNPs must be assessed on a case-by-case scenario.

The aim of the present study was to assess the effects of a particular formulation based on lipid and surfactants - Lipid Surfactant Submicron Particles (LSSPs) – loaded with melatonin at1 mg/mL, designed to be used as food additive. The formulation has been developed to be fully regulatory compliant for its potential use in the market and all components are food additives (EC 1333/2008).

The soil model species *Enchytraeus crypticus* (Oligochaeta) (OECD 220, 2016) and *Folsomia candida* (Collembola) (OECD 232, 2016) were used, covering two main life traits in soil and different exposure routes. Both species are secondary decomposers, but while collembolans live in the soil surface and soil porewater is a major exposure route, enchytraeids bury into soil and dietary exposure is the most relevant route. The effects were investigated 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), available and directly comparable for both species (Guimarães et al., 2019a; Ribeiro et al., 2018). This allows for a comparison between species at similar exposure periods and covering a longer-term exposure, as recommended for nanomaterials. Hence, the current study will not only contribute to providing data on a knowledge gap material area but also on a relevant biological aspect, where long term is seldom covered.

2. Materials and methods

2.1. Test organisms

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) and *Folsomia candida* (Collembola) were used as test species. Both were kept in cultures under controlled conditions: 20 ± 2 °C, and photoperiod of 16:8 h light:dark. Synchronized age organisms (18–20 days old after cocoon laying) were used.

The enchytraeids were cultured in agar, consisting of sterilized Bacti-Agar medium (Oxoid, Agar No. 1) and a 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 $NaHCO_2$. The animals were fed with ground autoclaved oats twice per week. Cultures were synchronized to obtain 18–20 days old organisms (the details on culture synchronization can be found in (Bicho et al., 2015)).

The collembolans were cultured on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio). The animals were fed with dried baker's yeast (*Saccharomyces cerevisiae*) once a week. Cultures were synchronized to obtain 10–12 days old juveniles (OECD 232, 2016).

2.2. Test soil

The natural standard LUFA 2.2 soil (LUFA Speyer, Germany) was used. Its main characteristics are: pH (0.01 M $CaCl_2$) = 5.6 ± 0.4; organic carbon = 1.71 ± 0.30 %; cation exchange capacity (CEC) = 9.2 ± 1.4 meq/100 g; maximum water holding capacity (maxWHC) = 44.8 ± 2.9 g/100 g; grain size distribution = 8.0 ± 1.5 % clay, 13.7 ± 1.0 % silt, and 78.3 ± 1.0 % sand.

2.3. Test materials and characterization

A water-based colloidal suspension of lipid-surfactant submicron particles – LSSP – containing melatonin, was produced based on adapted microemulsion method. The composition for LSSPs is reported in Table 1. The same formulation has been investigated avoiding addition of the thickening agent xanthan gum, usually present as stabilizer in water phase, designated as LSSP-xg. Sodium benzoate was used as preservative, at usual effective concentration, as by regulation. Formulation was obtained by stirring, without stressing the achievement to very small dimensions, targeting characteristics as allowed to avoid falling under Novel Food regulation.

$LSSP_S$ were characterized for the size/shape of the particles by Transmission Electron Microscopy (TEM), by using a JEOL-JEM 1010 microscope operating at an acceleration voltage of 100 kV. TEM images were acquired to measure the size and characterize nanomaterial morphology.

The hydrodynamic diameter was determined by Dynamic Light Scattering (DLS) and the ζ-potential by Electrophoretic light Scattering (ELS). Measurements were performed by using the Zetasizer Nano instrument (Malvern Instruments, Malvern, UK) with a 633 nm HeNe laser. Instrument settings: replicate 3, equilibrium time 60 s, $T = 25$ °C, dispersant refractive index 1.330, dispersant viscosity 0.8872 cP, material refractive index 1.54, material absorption 1.000. Melatonin concentration was evaluated by High Performance Liquid Chromatography (HPLC). A summary of the materials characteristics can be found in Table 1.

2.4. Spiking procedures

The tested concentrations were 0, 10, 50, 75, 100 mg/kg soil dry weight for both LSSPs and LSSPs-xg. For *F. candida* tests the additional concentration of 200 mg/kg soil was tested. Spiking followed the recommendations for nanomaterials (OECD, 2012), with each replicate prepared individually to ensure total raw amounts of the tested materials. Stock aqueous dispersions were prepared, serially diluted, and added to pre-moistened soil (20/40 g for *E. crypticus*, or 30 g for *F. candida*) to achieve 50 % of soil maxWHC. The soil was homogeneously mixed and was left to equilibrate for 1 day prior test start.

2.5. Test procedures

Toxicity assessment was done for the two test species based on the OECD standard tests (OECD 220, 2016; OECD 232, 2016) and the standard extension (Guimarães et al., 2019a; Ribeiro et al., 2018). The test procedures are detailed bellow, for the two species, separately. Endpoints included, in addition to the standard survival and reproduction (28 days), for *E. crypticus* survival at the intermediate times (7, 14, 21 days), size (28 days adults), and total number of animals (56 days), and for *F. candida*: survival and reproduction at the intermediate times (7, 14, 21 days), size (28 and 56 days for adults and juveniles), survival and reproduction at day 56 (2nd generation).

2.5.1. Enchytraeus crypticus

Tests with enchytraeids followed the standard guideline (OECD 220, 2016) (28 days), plus the OECD extension (56 days), as described in Ribeiro et al. (2018), as also performed several additional times (Gomes et al., 2023b, 2023a, 2022; Guimarães et al., 2022b; Hund-Rinke et al., 2021). In summary, the sampling times: endpoints were i) survival: 7, 14, 21, 28, 56 days; ii) reproduction: 28 and 56 days; iii) size: 28 and 56 days. Four replicates per treatment were done, except at days 7, 14 and 21 with 1 replicate. For full details on exposure procedures see Ribeiro et al. (2018), and for details on size determination see e.g. (Gomes et al., 2023b). Test ran at 20 ± 1 °C and 16:8 h photoperiod. Food (11 ± 1 mg: until day 28, and 33 ± 3 mg: from 28 to 56 days) and water were replenished weekly. The adult organisms collected at day 28 were photographed, and size (size, mm) was assessed using the software ImageJ (v.1.52a, Wayne Rasband, National Institutes of Health, USA).

2.5.2. Folsomia candida

Tests with collembolans followed the standard guideline (OECD 232, 2016) (28 days) plus the OECD extension (56 days), representing one more generation compared to the standard, as described in Guimarães et al. (2019a), and as performed several additional times (Gomes et al., 2023b; Guimarães et al., 2022a, 2022b, 2019b; Hund-Rinke et al., 2021). In summary, the sampling times: endpoints were i) survival and reproduction: 7, 14, 21, 28, 56 days; and ii) size: 28 and 56 days. Four replicates per treatment were done, except at days 7, 14 and 21 with 1 replicate. For details on test procedures see Guimarães et al. (2019a). Test ran at 20 ± 1 °C, under a 16:8 h photoperiod. Food supply (2–10 mg, baker's yeast) and water was replenished weekly. At each sampling day, test vessels were flooded with water, and the surface was photographed in a crystallizer dish for further analyses (count and measure (size, area)) using the software ImageJ (v.1.52a, Wayne Rasband, National Institutes of Health, USA). For the 56 days exposure replicates, at day 28 the sampled juveniles, 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.

2.6. Data analysis

The differences between controls and treatments were assessed, for all the endpoints, using One-way analysis of variance (ANOVA), followed by the Dunnets' Post-Hoc test (SigmaPlot v.14.0, Systat Software, Inc., San Jose California USA). Effect concentrations (ECx) were calculated, for the various endpoints, modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 2, using the Toxicity Relationship Analysis Program software (TRAP 1.30a, USEPA).

Table 1
Summary of the composition and properties of LSSPs (Lipid Surfactant Submicron Particles) and LSSPs-xg (the same LSSPs but without final addition of stabilizer xanthan gum).

Material	LSSPs	LSSPs-xg
Excipients (in order of decreasing %)	Water, glycerol (8–10 %), dibasic sodium phosphate (1.4 %), citric acid (0.8 %), lecithin (soy), mono-diglycerides esterified with citric acid/lactic acid, sodium benzoate, polysorbate 20, glyceryl monostearate, ascorbyl palmitate, alpha tocopherol acetate, strawberry flavor, sucralose	
Stabilizer	Xanthan gum	–
Active substance[a] (0.1 % w/w)	Melatonin (1.03 mg/mL)	Melatonin (1.00 mg/mL)
Dispersant medium	Citrate/phosphate buffer pH 5	
Mean hydrodynamic diameter[b] (Z-average, nm)/PDI	238.6/0.291	135.0/0.244
ζ-potential (mV)[c]	−41.0	−16.3

[a] High Performance Liquid Chromatography (HPLC).
[b] Dynamic light scattering (DLS).
[c] Electrophoretic light scattering, (ELS); PDI: polydispersity index.

Table 2
Summary of the effect concentrations (ECx with 95 % confidence intervals – CI), expressed as mg of formulation per kg soil (dry weight), for *Enchytraeus crypticus* and *Folsomia candida* exposed to LSSPs (Lipid Surfactant Submicron Particles) and LSSPs-xg (LSSPs without stabilizer xanthan gum) in LUFA 2.2 soil. Log2P: logistic 2 parameters; Thres2P: threshold sigmoid 2 parameters; S: slope; Y0: top point; n.e.: no effect; n.d.: not determined.

Test species	Endpoint	Time (days)	EC10 (95 % CI)	EC50 (95 % CI)	EC90 (95 % CI)	Model & parameters
LSSPs						
E. crypticus	Survival	28	26 (25–27)	30 (29–31)	34 (33–35)	Log2P; S:0.15, Y0:10; r^2:1
	Reprod.	28	11 (−15–36)	15 (−161–192)	19 (−308–348)	Log2P; S:0.12, Y0:896, r^2:0.96
	Total org.	56	28 (−1488–1544)	34 (−1083–1151)	40 (−680–760)	Log2P; S:0.095, Y0:3372, r^2:0.95
	Size	28	n.e.	n.e.	n.e.	–
F. candida	Survival	28	41 (−4–87)	55 (53–58)	64 (−10–139)	Thres2P; S:0.04, Y0:10, r^2:0.97
	Reprod.	28	36 (−366–439)	44 (−123–211)	52 (−16–121)	Log2P; S:0.068, Y0:1022, r^2:0.98
	Survival	56	54 (50–57)	61 (−423–545)	66 (63–69)	Thres2P; S:0.072, Y0:9.4, r^2:0.99
	Reprod.	56	44 (−247–336)	51 (14–88)	57 (−308–422)	Log2P; S:0.087, Y0:990, r^2:0.97
	Size-adults	28	n.d.	n.d.	n.d.	–
	Size-juv.	28	23 (1–44)	72 (53–91)	103 (63–143)	Log2P; S:0.011, Y0:0.116, r^2:0.65
	Size-adults	56	n.e.	n.e.	n.e.	–
	Size-juv.		n.e.	n.e.	n.e.	–
LSSPs-xg						
E. crypticus	Survival	28	11 (−4–26)	34 (25–44)	49 (34–63)	Thres2P; S:0.023, Y0:10, r^2:0.88
	Reprod.	28	14 (−45–72)	29 (21–36)	38 (−421–497)	Thres2P; S:0.037, Y0:896, r^2:0.97
	Total org.	56	9 (3–16)	14 (−32–61)	19 (−80–118)	Log2P; S:0.11, Y0:3234, r^2:0.97
	Size	28	n.e.	n.e.	n.e.	–
F. candida	Survival	28	38 (23–52)	52 (48–55)	66 (48–83)	Log2P; S:0.039, Y0:9.75, r^2:0.95
	Reprod.	28	38 (−6493–6569)	44 (−3243–3331)	50 (7–92)	Log2P; S:0.092, Y0:1002, r^2:0.98
	Survival	56	48 (33–64)	55 (13–96)	61 (−37–159)	Log2P; S:0.085, Y0:9.63, r^2:0.98
	Reprod.	56	45 (−175–265)	51 (−1–104)	57 (−268–383)	Log2P; S:0.088, Y0:1025, r^2:0.96
	Size-adults	28	44 (−687–775)	54 (−433–541)	64 (−1642–1769)	Log2P; S:0.056, Y0:1.51, r^2:0.89
	Size-juv.	28	23 (−5–51)	59 (44–75)	96 (44–147)	Log2P; S:0.015, Y0:0.118, r^2:0.65
	Size-adults	56	51 (37–65)	69 (−211–349)	88 (−460–636)	Log2P; S:0.03, Y0:1.64, r^2:0.4
	Size-juv.	56	46 (−210–302)	58 (−460–577)	71 (−1223–1364)	Log2P; S:0.044, Y0:0.13, r^2:0.6

3. Results

3.1. Materials characterization

The LSSPs samples are water-based colloidal suspensions. Preservative, stabilizers, surfactants, antioxidants and salts are contained to prolong the shelf-life of the product. LSSPs and LSSPs-xg have the same composition, except for the presence in LSNPs of xanthan gum as stabilizer (Table 1), that prevents precipitation.

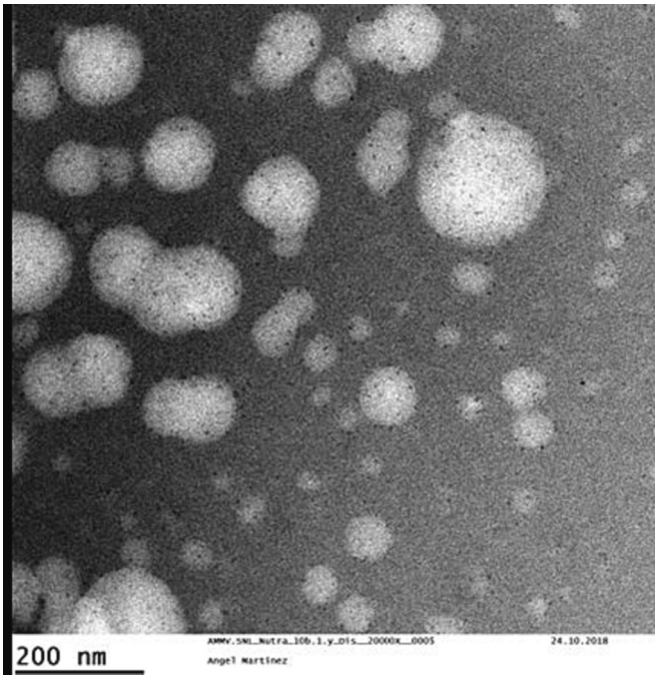

Fig. 1. LSSPs (Lipid Surfactant Submicron Particles) images from Transmission Electron Microscopy (TEM).

Both preparations come from same precursor with a mean hydrodynamic diameter of 238.6 nm, and 135 nm respectively. The Polydispersity Index (PDI) is compatible with a polydisperse colloidal suspension (Table 1). TEM images of LSSPs (Fig. 1) shows particles having a large range of diameters, in agreement with the DLS data, and the presence of agglomerates/aggregates (Fig. S1). ζ-Potential was negative, confirming negative surface charge as expected by the used components, LSSPs had a more negative value, due to the presence of the stabilizer.

3.2. Ecotoxicological tests

For the *E. crypticus* tests, the validity criteria were fulfilled (OECD 220, 2016), i.e., in controls, adult mortality was below 20 % and the number of juveniles was higher than 50 per replicate, with a coefficient of variation lower than 50 %.

Results showed that both LSSPs and LSSPs-xg caused toxic effects in terms of survival and reproduction from 50 mg/kg soil and above. In the case of LSSPs, the effect is 100 % mortality (Fig. 2). The adults' size was not affected but measurements were only possible at 10 mg/kg due to mortality in all other treatments (see Supplementary material Fig. S2).

Reproduction was slightly more sensitive than survival, although the confidence intervals overlap (Table 2). The EC50 were similar at 28 and 56 days, being in terms of survival_28 days, reproduction_28 days and total organism_56 days the following: 30, 15 and 34 mg LSSPs/kg soil and 34, 29 and 14 mg LSSP-xg/kg.

For *F. candida* tests, the validity criteria were fulfilled (OECD 232, 2016), i.e., in controls, adults' mortality was below 20 % and number of juveniles was higher than 100 per replicate, with a coefficient of variation lower than 30 %.

The results showed that both LSSPs and LSSPs-xg caused toxic effects in terms of survival and reproduction at 50 mg/kg soil and above, being of 100 % mortality at 75 mg/kg and above (Fig. 3A). The exposure for a 2nd generation (56 days) showed less pronounced effects, both in terms of survival and reproduction (Fig. 3B). This is easy to notice in the results

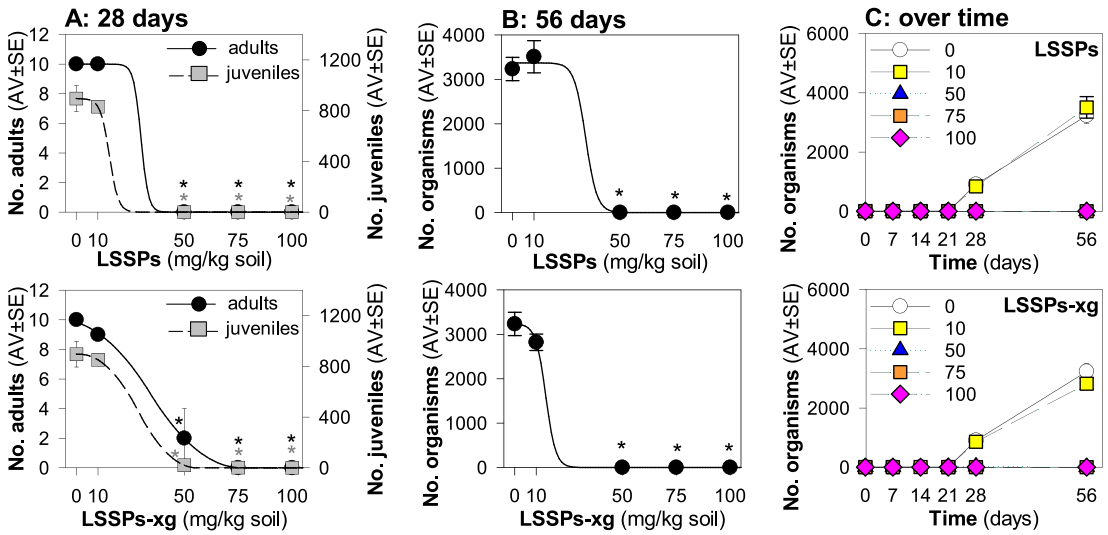

Fig. 2. Results in terms of survival and reproduction when exposing *Enchytraeus crypticus* in LUFA 2.2 soil to LSSPs (Lipid Surfactant Submicron Particles) and LSSPs-xg (LSSPs without stabilizer xanthan gum) during (A) 28 days (OECD Standard), (B) 56 days (OECD standard extension), and (C) overview of the time series sampling at days: 7, 14, 21, 28 and 56 days. Values represent number of adults, juveniles, and population as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's).

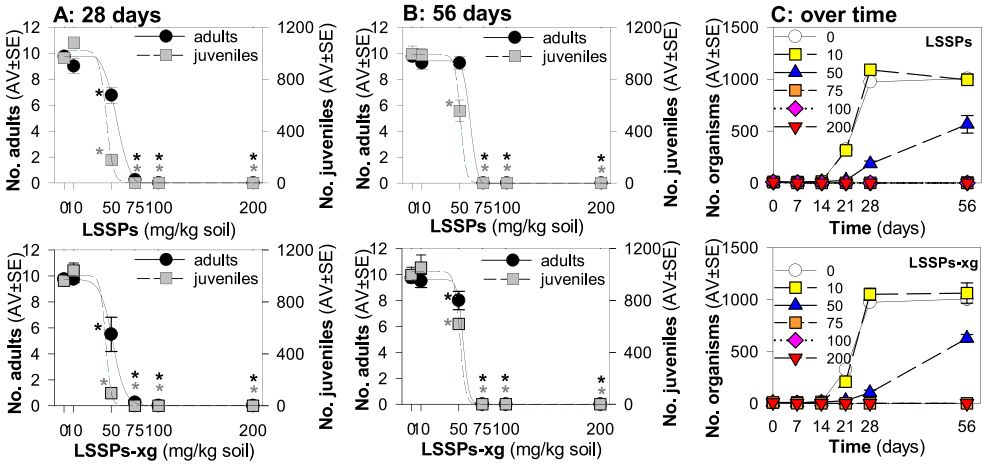

Fig. 3. Results in terms of survival and reproduction when exposing *Folsomia candida* in LUFA 2.2 soil to LSSPs (Lipid Surfactant Submicron Particles) and LSSPs-xg (LSSPs without stabilizer xanthan gum) during (A) 28 days (OECD Standard), (B) 56 days (extension for 2nd generation), and (C) overview of the time series sampling at days: 7, 14, 21, 28 and 56 days. Values represent number of adults, juveniles, and population as average ± standard error (AV ± SE). *: $p < 0.05$ (Dunnett's).

over time (Fig. 3C), where an increase in the number of organisms is observed at 50 mg/kg soil, for both LSSPs and LSSPs-xg, from 28 to 56 days.

The difference is small and reflected in the similar ECx, e.g., reproduction_EC50 of 44 and 51 mg LSSPs/kg soil for 28 and 56 days, respectively; the same for LSSPs-xg, and with overlapping confidence intervals (Table 2). Size was more affected by LSSPs-xg than by LSSPs (Fig. 4).

LSSPs caused a dose-dependent decrease in the size of juveniles exposed for 28 days (size_EC50 = 72 mg/kg), while LSSPs-xg caused a reduction in the size of adults and juveniles exposed for both generations (28 and 56 days).

4. Discussion

LSSPs in its media was toxic to the soil invertebrates *E. crypticus* and *F. candida*, reducing their survival and reproduction in a dose-dependent way. Similar effects were observed for LSSPs-xg showing that xanthan gum is not likely the main cause for the toxicity observed.

The ECx reported here for LSSPs and LSSPs-xg based on the OECD standard 28 days tests (*E. crypticus*: LC50 = 30 mg/kg, EC50 = 15 mg/kg; *F. candida*: LC50 = 55 mg/kg, EC50 = 44 mg/kg) are in a much lower range than those reported previously for other NMs. For instance, for metallic NMs, silver (Ag) (*E. crypticus* Ag NM300K_LC50 = 657 mg Ag/kg, Ag NM300K_EC50 = 161 mg/kg (Bicho et al., 2016); *F. candida* Ag NM300K_LC50 > 640 mg Ag/kg, Ag NM300K_EC50 = 540 mg Ag/kg (Mendes et al., 2015)), or carbon based NMs (*E. crypticus* graphene oxide_LC50 = 447 mg/kg, graphene oxide_EC50 = 740 mg/kg, based on a full life cycle test (Mendonça et al., 2019); *F. candida* multi walled carbon nanotubes_LC50/EC50 > 6400 mg/kg (Noordhoek et al., 2018)). In fact, the toxicity reported here is in the same range as reported for certain pesticides, such as the fungicide azoxystrobin to *E. crypticus* (LC50 = 39 mg/kg, EC50 = 37 mg/kg (Gomes et al., 2021)) or the veterinary parasiticide ivermectin to *F. candida* (LC50 = 40 mg/kg, EC50 = 5 mg/kg (Guimarães et al., 2019a)).

The toxicity of similar particles and their media based on food additives to soil invertebrates is unknown: this formulation is based on amphiphilic lipids, with absence of pure fat phase as by composition reported above, and there are not any pure lipids. Those "soft matter" assemblies are different from inorganic particles reported above and can

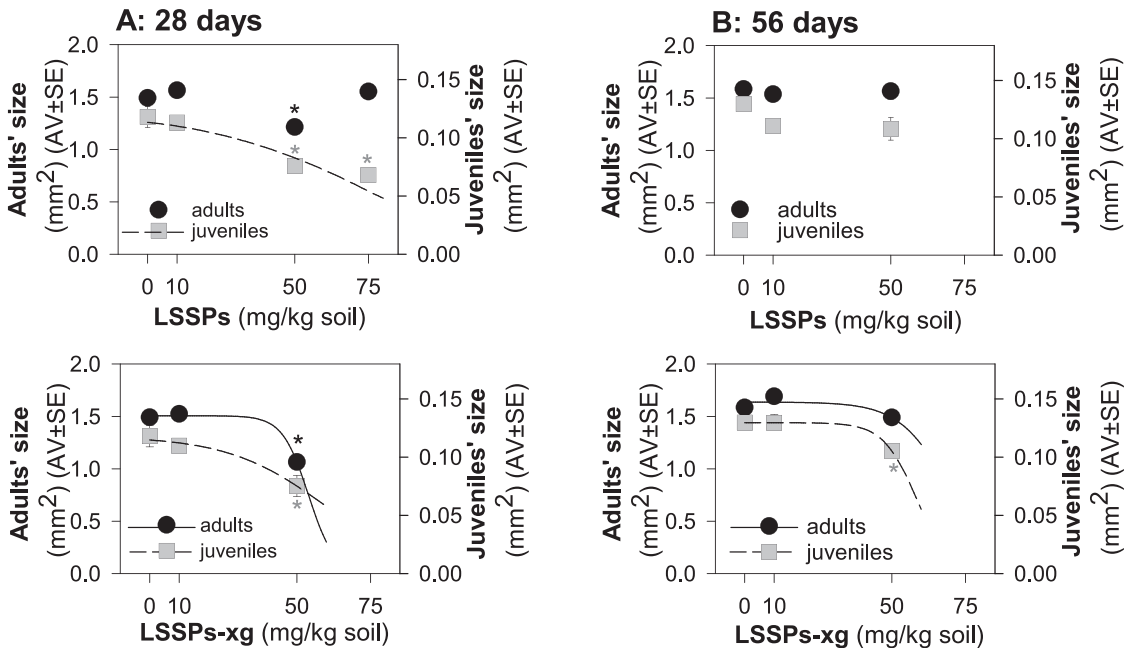

Fig. 4. Results in terms of organisms' size when exposing *Folsomia candida* in LUFA 2.2 soil to LSSPs (Lipid Surfactant Submicron Particles) and LSSPs-xg (LSSPs without stabilizer xanthan gum), during A) 28 days and B) 56 days (extension for 2nd generation). Values represent adults' and juveniles' size (area, mm^2) as average ± standard error (AV ± SE).

release many different components of amphiphilic nature which can have strong interaction with living organism. All the components of the formulation are food grade, thus high environmental impacts would not be expected. For instance, ecotoxicity studies with glycerol (which accounts for 8–10 % of the total formulation) on aquatic organisms: the freshwater crustacean *Daphnia magna* (Perales et al., 2017) and the marine bacteria *Vibrio fischeri* (García et al., 2015), showed no toxicity (EC50 > 10,000 mg/L). Citric acid (0.8 % of the formulation) was used to remediate cadmium contaminated soil, improving soil's bacterial community (Ma et al., 2020). However, the effects of the mixture/formulation cannot be predicted.

Overall *E. crypticus* was more sensitive to both LSSPs, since *F. candida* was still able to survive and reproduce at 50 mg/kg, even though significant adverse effects were already observed. Further, while for *E. crypticus*, the effects on reproduction are associated with decreased survival of the adults, for *F. candida* reproduction was slightly more sensitive than survival, which is often reported (e.g. for silver (Mendes et al., 2015), for cadmium (Guimarães et al., 2019c), for ivermectin (Guimarães et al., 2019a)). Size was only affected for concentrations that affected survival and reproduction, hence a less sensitive endpoint. It is of course not possible to assess the impact on size at concentrations where survival was 0 %.

For *E. crypticus*, the toxicity patterns of LSSPs and LSSPs-xg were maintained after 56 days of exposure, indicating that a concentration up to 10 mg/kg LSSPs is not toxic even at longer-term exposure. For *F. candida*, the organisms exposed for a second generation seemed to be less affected by both LSSPs, i.e., less severe effects were observed for 50 mg/kg, at 56 days in comparison to 28 days, considering all the endpoints – survival, reproduction, and size. This could be partly due to the implemented design, in which the 10 largest juveniles were selected for exposure in the next generation, although the same design has shown increased impact in the consequent generations, e.g. (Guimarães et al., 2022a, 2019b).

A study on the same lipid surfactant particles tested here (LSSPs), showed low to no toxicity to epithelial intestinal cells (Caco-2,HCT116 and HCoEpiC cells), although the toxicity increased after the treatment of the particles with simulated human digestive system (SHDS) (Antonello et al., 2022). The increase in toxicity was explained as caused by the degradation of the outermost layers' and the release of the surfactants, induced by the SHDS (Antonello et al., 2022). Generally lipid based NPs are considered safe nanocarriers for medicines, without significant effects reported in vivo, mostly in rodent models, as reviewed by Doktorovová et al. (2016). However, the potential of some lipid-based nanostructures to induce genotoxic effects was highlighted by Azarnezhad et al. (2020). Our current results show that even formulations based on food additives can show toxicity to two non-target soil species, raising concern about the environmental effects of other lipid-surfactant based nanostructures. One study has shown that the in vitro cytotoxicity to RAW264.7 cells of lipid nanocapsules of different sizes (25, 55 and 100 nm) – empty from any drug – was probably caused by one of the surfactants present in the formulation (Le Roux et al., 2017). Also, the degradation of the outermost layers' of lipid-surfactant nanoparticles and release of the surfactants, induced by the treatment of the particles in a simulated human digestive system, were probably the responsible of the toxicity observed to epithelial intestinal cell lines (Antonello et al., 2022). The active substance in LSSPs is melatonin and its toxicity has not been investigated in soil invertebrates. Melatonin is a ubiquitous molecule present in animals, plants, fungi, and bacteria, and thus not expected to cause toxicity. In plants, melatonin is a growth biostimulator, capable of minimizing possible harmful effects through the control of reactive oxygen species (ROS) levels and activating antioxidative responses (B. Arnao and Hernández-Ruiz, 2019).

While lipid-based NPs used in pharmaceutical products are not expected to impact the environment, given the low volumes and regulated pathways for waste, the present study shows that even additives usually used for food or cosmetic applications can generate unexpected toxicity in soil invertebrate models suggesting the need of further in-depth investigation to understand whether it is their particular assembly in a particular media (whole formulation), or their pure nature that cause this.

5. Conclusions

Lipid surfactant submicron particles (LSSP) loaded with melatonin, and in their functional media, pH buffer, preservatives, etc., were toxic to soil invertebrate models (enchytraeids and collembolans). Negative effects were observed in all the endpoints assessed, i.e., reduction of survival, reproduction (and size for collembolans) in a dose-dependent way. Reproduction was the most sensitive endpoint, with 28-day exposure EC50 = 15 and 44 mg LSSPs/kg soil for *E. crypticus* and *F. candida*, respectively. These values are in the same range as reported for some classes of pesticides, highlighting the potential environmental implications for enchytraeids and collembolans, for which populations can be at risk if these materials reach the environment. There were no indications of increased toxicity with longer exposure period. Toxicity could be related to many of the different components of the formulation. The testing of the different ingredients of the formulation would be recommended to further detail and understand the source of the observed toxicity.

CRediT authorship contribution statement

Susana I.L. Gomes: Writing – review & editing, Writing – original draft, Methodology, Data curation. **Bruno Guimarães:** Methodology. **Ivana Fenoglio:** Writing – review & editing, Conceptualization. **Paolo Gasco:** Writing – review & editing, Resources, Conceptualization. **Ana Gonzalez Paredes:** Writing – review & editing, Methodology, Investigation. **Magda Blosi:** Writing – review & editing, Investigation, Conceptualization. **Anna L. Costa:** Writing – review & editing, Investigation, Conceptualization. **Janeck J. Scott-Fordsmand:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Data curation, Conceptualization. **Mónica J.B. Amorim:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169748.

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