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On the safety of nanoformulations to non-target soil invertebrates – an atrazine case study†

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The use of nanotechnology in the agrochemical sector aims to increase pesticide efficiency, and at the same time provide more targeted delivery, reducing the application volume and thus its environmental footprint. However, the possible risks of these new nanopesticides to non-target organisms are still sparsely investigated. The aim of the present study was to investigate the effects of a nanoformulation of atrazine (nano_ATZ) to non-target soil invertebrates. The effect was compared with the commercial formulation (Gesaprim®) and atrazine (the pure active ingredient, a.i.), using the a.i. in a field concentration range using the soil invertebrate, *Enchytraeus crypticus* (Oligochaeta) as the non-target organisms. The endpoints evaluated included avoidance behaviour (2d), hatching success (11d), survival and reproduction (based on both the standard enchytraeid reproduction test (28d) and on the full life cycle test (46d)). Results showed that enchytraeids avoided soil spiked with Gesaprim and atrazine (a.i.), but not nano_ATZ. While all tested atrazine forms affected the hatching success (11d, early development stage), the toxicity in later stages, as measured in terms of survival and reproduction (46d) showed that Gesaprim was the least toxic (EC10 *ca.* 200 mg kg^{-1}), followed by nano_ATZ (EC10 *ca.* 180 mg kg^{-1}) and atrazine (a.i.) (EC10 *ca.* 100 mg kg^{-1}). These findings are important to nanopesticide regulatory purposes, showing the potential effects of nanoformulation compared to the current commercial non-nano ATZ in a.i. field concentrations, and that information on additional test species and exposure routes are missing, as well as the longer term consequences.

Environmental significance

For agrochemicals only a small fraction of the applied reaches the target organisms and even less the target-site within the organisms. Nanoagrochemicals aim to increase pesticide efficiency by providing more targeted delivery allowing for a reduction of the application volume. One such nanoagrochemical is the nanoformulation of atrazine, which can be 10 times more efficient toward target species than normal products. However, the possible non-target effects of nanoagrochemicals are unknown. We found that when exposing the non-target species *Enchytraeus crypticus* to a nanoformulation containing atrazine, "free" atrazine and a commercial formulation of atrazine, the commercial formulation was the least toxic followed by the nanoformulation and the free atrazine. This illustrates the need for an evaluation of benefits (targets) *versus* risks (non-target).

Introduction

Nanotechnology research on applications in the agrochemical sector has increased substantially over the past decade,[1] particularly in terms of plant-protection products. The use of nanoencapsulation technology (*i.e.*, the coating of various substances by another material, *e.g.*, polymers or lipids, to produce structures with sizes in the nano-range) has been applied to commercial pesticides, promising increased efficiency in terms of environmental stability, controlled release, target activity, and physical stability compared to other formulations.[2] Nevertheless, a recent review[3] highlighted the insufficient data to support the overall concept of agrochemical efficacy gained from nano-enabled products.

Most of the data generated so far have suggested that the use of nano-encapsulated pesticides is less harmful to cell lines or non-target organisms than the pure active ingredients (a.i.s). For instance, polymeric-nanoparticles loaded with the herbicide metolachlor (a.i.) showed effective herbicidal activity against *Oryza sativa*, *Digitaria sanguinalis* and *Arabidopsis thaliana*, and lower cytotoxicity than that

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observed with metolachlor (a.i.) to the MC3T3 cell line.[4] Also, Grillo *et al.*[5] showed that the polymeric-nanocapsule formulations of ametryn, atrazine, and simazine induced less DNA damage to human lymphocytes than the corresponding herbicides (pure a.i.s). Using the same polymeric-nanocapsules containing the herbicide atrazine (a.i.), Oliveira *et al.*[6] showed that they do not cause persistent effects on maize plants but did cause effects on mustard plants. However, nanoformulations (including polymeric-nanocapsules, solid-lipid nanoparticles and chitosan/tripolyphosphate nanoparticles) of atrazine/simazine, atrazine, and paraquat (a.i.s) were more toxic to the nematode *Caenorhabditis elegans* (*in vivo*) than the respective a.i.s.[7] This highlights the need for further research to fully investigate the environmental hazard of nanoformulations, particularly concerning whether nanoformulations can enhance species- or group-specificity and sensitivity, which will also reduce application loads. Further, if there are few studies comparing the activity of a nanoformulation to that of the pure a.i. and the commercial (non-nano) formulation,[3] there are even fewer studies comparing the effects on non-target organisms.

Currently, there is very little information regarding the toxicity of nanoformulations to non-target organisms, in particular for soil living organisms (including invertebrates) which are among the first in line to be exposed to agrochemicals. The aim of the present study was to investigate the effects of a nanoformulation of atrazine (atrazine encapsulated inside polymeric nanocapsules), in comparison with atrazine (pure a.i.) and a commercial formulation (Gesaprim® 500 CG, 50% m/v atrazine a.i.) using a.i. concentrations in a field range. Atrazine was chosen since it is relatively well understood and still used in large parts of the world. Effects were assessed on the non-target organism *Enchytraeus crypticus* (Oligochaeta), a soil invertebrate. *E. crypticus* is a standard species in soil ecotoxicology[8] with a vast array of additional endpoints available, including avoidance and full life cycle tests,[9,10] besides covering several omics.[11–13] In the present study, in addition to the standard 28 day enchytraeid reproduction test (ERT) to assess survival and reproduction, the effects were assessed in terms of avoidance behaviour (2 days), cocoon hatching (11 days) and after longer-term exposure (survival and reproduction after 46 days of exposure of the full life cycle test (FLCt)). The concentrations tested (1 to 400 mg atrazine per kg soil) and effect levels (ECx) observed (see later), are within the relevant field concentrations of atrazine (*e.g.* measurements detected *ca.* 6 mg atrazine per kg soil immediately after field use application, in the top 10 cm of soil[14]) and the soil quality criteria in various areas are 22 mg atrazine per kg.[15]

Materials and methods

Preparation of polymeric nanocapsules

The nanocapsules were prepared by the nanoprecipitation method, involving the mixing of an organic phase in an aqueous phase.[5] The organic phase consisted of the poly(ε-caprolactone) (PCL) polymer (100 mg), acetone (30 mL), Span® 60 (sorbitan stearate, used as detergent) (20 mg), Myritol® (mixed decanoyl and octanoyl triglycerides, used as emollients) (200 mg) and atrazine (10 mg). The aqueous phase was composed of Tween® 80 (polysorbate 80, used as non-ionic surfactant) (60 mg) and deionized water (Milli-Q, Millipore) (30 mL). The organic phase was poured into the aqueous phase. The resulting suspension was kept under stirring for 10 min and then concentrated under low pressure to a volume of 10 mL with the aid of a rotary evaporator to a final concentration of 1 mg atrazine per mL. Additionally, labelled-polymeric nanocapsules were synthesized to trace the uptake in the worms. For the labelled nanocapsules, 0.1% over the lipid mass of the probe Liss Rhod Avanti PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) – Polar Lipids®) was added to the organic phase and the entire system was protected from light. The rest followed the protocol as previously described.

Nanoparticle characterization

Photon correlation spectroscopy and microelectrophoresis techniques were used to determine the hydrodynamic diameter and zeta potential of the nanocapsules, respectively. The samples were diluted with water (Milli-Q) and analyzed using a ZetaSizer ZS 90 (Malvern®) at a fixed angle of 90° and temperatures of 25 °C. The concentrations and size distribution of the nanocapsules containing atrazine were analyzed using the nanoparticle tracking analysis (NTA) technique. Data were collected using a NanoSight LM 10 cell (532 nm) and a sCMOS camera using NanoSight software (version 3.1). The nanocapsule suspensions were diluted (5000 times), and triplicate analyses were performed for each sample. To ensure that different particles were analysed, for each replicate, 1 mL of the sample suspension was injected into the volumetric cell in order to displace the previously measured content. In addition, the morphology of the nanocapsules was evaluated by scanning electron microscopy (SEM, EVO-LS-15, Carls Zeiss), operated at 15 kV of high voltage with a spot size between 3.0 and 4.0 and a working distance (WD) of 10.0 mm.

Test organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta), Westheide & Graefe, 1992, 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 hours light : dark). The cultures were fed with ground autoclaved oats twice per week.

Test soil

The natural standard LUFA 2.2 soil (Speyer, Germany) was used. Its main characteristics are: pH (0.01 M $CaCl_2$) = 5.5;

organic carbon = 1.61%, cation exchange capacity (CEC) = 10.0 meq/100 g, maximum water holding capacity (maxWHC) = 43.3%, and a grain size distribution of 7.9% clay (<0.002 mm), 16.3% silt (0.002–0.05 mm), and 75.8% sand (0.05–2.0 mm).

Test chemicals and spiking

Gesaprim® 500 CG (Syngenta, 50% m/v atrazine) was purchased from local suppliers. Atrazine (Pestanal, analytical grade, >98%) was purchased from Sigma-Aldrich, and it is the a.i. of Gesaprim and is further referred to as ATZ. Polymeric nanocapsules containing atrazine (further referred to as nano_ATZ) and polymeric capsules alone, to serve as a control (further referred to as NCs), were prepared as described above. The tested concentrations for Gesaprim were 0–1–5–10–50–100–200–400 mg ATZ per kg soil dry weight (DW) and 0–1–5–10–50–100–200 mg ATZ per kg DW for the ATZ and nano_ATZ. Gesaprim is water soluble, so it was serially diluted and added to the pre-moistened soil (batches of soil, per concentration). The soil was homogeneously mixed and deionised water was added until 50% of soil's maxWHC. The soil was mixed again, divided into each test vessel, and was allowed to equilibrate for 1 day prior to the start of the test.

Atrazine (ATZ) was dissolved in acetone, due its low solubility in water, and serially diluted to the desired test concentrations (as stated above), 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 test started immediately thereafter.

For nano_ATZ, the stock (aqueous) suspension was serially diluted and added to the pre-moistened soil, with each replicate prepared individually (to ensure total raw amounts of the tested material). The soil was homogeneously mixed and deionised water was added until 50% of soil's maxWHC. NC controls (containing the polymeric nanocapsules without ATZ) were prepared using NC (aqueous) dispersions. Soil was allowed to equilibrate for 1 day prior to the start of the test.

Test procedures

Avoidance tests. Avoidance tests were performed following the earthworm avoidance test guideline[16] using *E. crypticus* with adaptations as described in ref. 9. 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 this, 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 worms. For the Gesaprim test, the control consisted of moist (50% maxWHC) LUFA 2.2 soil. For the ATZ test, the control for each comparison was the solvent control; an additional solvent control *versus* moist LUFA 2.2 soil test was performed to assess the possible effects of acetone. For the nano_ATZ test, each test condition was performed *versus* the respective NC control (*e.g.*, for the concentration of 50 mg ATZ per kg of nano_ATZ, the control was the NC suspension at the same dilution); an additional NC stock suspension *versus* moist LUFA 2.2 soil test was performed.

Reproduction tests. The enchytraeid reproduction test (ERT) procedures followed the OECD guideline[8] with adaptations. In short, 10 18 d old age-synchronized individuals (for culture synchronization see ref. 10) were introduced in each test vessel containing 20 g of moist soil and 25 mg of food (autoclaved ground oats). This test ran for 28 d at 20 ± 1 °C and a photoperiod of 16 : 8 h (light : dark). During the test duration, food (12 mg) and water contents (based on weight loss) were replenished weekly. Four replicates per treatment were used, including controls (1: LUFA 2.2 soil moistened to 50% maxWHC; 2: solvent control for ATZ test; 3: NC control, equivalent to the concentration of 200 mg ATZ per kg for the nano_ATZ test). At the end of the test, the organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, the soil samples were sieved through meshes with a decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction were assessed.

Full life cycle tests (hatching, growth, survival and reproduction). A reduced version of the full life cycle test (FLCt), as described in the study of Bicho *et al.*,[10] was performed. Endpoints assessed included hatching success and juveniles' length (day 11), survival, reproduction and adults' length (day 46). In short, the test starts with cocoons (1–2 d old) selected from synchronized cultures. Ten cocoons were introduced in each test vessel (ø 40 mm, 7.5 cm height) containing 10 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 plus time point were used, including controls (1: LUFA 2.2 soil; 2: solvent control for the ATZ test; 3: NC controls, equivalent to the concentrations of 50 and 200 mg ATZ per kg for the nano_ATZ test). Food (6 mg autoclaved ground oats) was added for the first time at day 11 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 sub-sample of the organisms in each replicate (n = 20) was measured for length.

Uptake traceability assessment characterisation. Organisms were exposed to labelled_nano_ATZ using the FLCt design in a similar parallel additional experiment. Organisms were exposed to 0–100–200 mg ATZ per kg (DW) of labelled_nano_ATZ from the cocoon stage (1–2 d old). The test ran at 20 ± 1 °C

in the dark (the vessels were covered with aluminium foil to avoid contact with light and consequent fluorescence loss). Food (6 mg autoclaved ground oats) was added for the first time at day 11 and then replenished weekly together with water content (based on weight loss). The samples were collected at 7, 13, 25 and 46 d, under a stereomicroscope. The cocoons/organisms were washed with distilled water and mounted onto microscope slides, prior to observation with a fluorescence microscope (Zeiss Axio Imager Z19, with AxioCam HR).

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 ERT and FLCt tests, the controls (water and solvent, or water and NC controls) were compared using the *t*-test (for the ATZ test) or one-way analysis of variance (ANOVA) for the nano-ATZ test, at a significance level of 0.05. As there were no significant differences between controls, they were pooled prior to the performance of ANOVA, followed by the post-hoc Dunnett's method (for multiple comparisons) to assess the differences between test treatments and control, at a significance level of 0.05 (SigmaPlot 11.0).

Effect concentrations (ECx) were calculated, for various endpoints, modelling data to logistic or threshold sigmoid 2 or 3 parameter regression models, as indicated in Table 2, using the Toxicity Relationship Analysis Program (TRAP 1.30) software. Avoidance data were inverted to apply the regression models. For Gesaprim, variables were log transformed.

Results

Physicochemical characterization of the nanoformulations

The physicochemical properties of the polymeric nanocapsules (NCs) and nanocapsules containing atrazine (nano_ATZ) were evaluated immediately after preparation. A monomodal particle size distribution and a spherical particle morphology were observed, as shown in Fig. 1.

Table 1 summarizes the physicochemical characteristics of the nanoformulations, including values of mean diameter (MD), zeta potential (ZP), polydispersity index (PDI) and particle concentration (CT).

We also evaluated the mean diameter of nanocapsules containing ATZ (nano_ATZ) by DLS after serial dilutions (see Table S1, ESI†). Size distribution results showed that the suspensions containing the herbicide (nano_ATZ) have a diameter of 230–250 nm, and the suspensions of the nanocapsules alone (NCs) are slightly smaller, around 220–230 nm diameter (Table S1†). These results are in good agreement with those reported by Grillo *et al.*[5] It was also shown that the serial dilutions (within the concentration range tested) did not affect the size distribution of the particles (Table S1†).

Table 1 Characterization of polymeric nanocapsules (NCs) and nanocapsules containing ATZ (nano_ATZ), labelled (_L) or not: mean diameter (MD); polydispersity index (PDI); zeta potential (ZP) and concentration of particles (CT) using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) techniques. The values represent the means of three determinations

Formulation	MD (nm)	PDI	ZP (mV)	CT (10^{13} particles per mL)
NCs	233 ± 3	0.099 ± 0.02	−32.5 ± 0.8	0.81 ± 0.07
NCs_L	237 ± 7	0.122 ± 0.05	−32.3 ± 0.3	2.79 ± 0.11
nano_ATZ	236 ± 9	0.114 ± 0.04	−33.3 ± 1.1	0.84 ± 0.03
nano_ATZ_L	225 ± 3	0.160 ± 0.02	−33.1 ± 0.3	1.59 ± 0.07

Avoidance response

The results on avoidance response are shown in Fig. 2. The validity criteria were fulfilled, *i.e.*, less than 20% mortality and homogeneous distribution (no avoidance) in controls. There were no significant differences between the controls: control (unspiked soil) *versus* control_NCs, in the nano_ATZ, and control *versus* control_acetone, in the ATZ test, thus controls were pooled.

For nano_ATZ, there was no significant avoidance of the spiked soil. For ATZ, organisms avoided the spiked soil in a dose-dependent way, with significance (higher than 80% response) at 200 mg ATZ per kg. For Gesaprim, there was more than 50% avoidance from 50 mg ATZ per kg; all the ECx were estimated (Table 2).

Enchytraeid reproduction test (ERT)

The results on adults' survival and juveniles' production are shown in Fig. 3 and the ECx calculated are shown in Table 2. The validity criteria were fulfilled, *i.e.*, in controls, adult mortality was below 20% and the number of juveniles was higher than 50, with a coefficient of variation lower than 50%. There were no significant differences between the control and control_NCs or the control and control_acetone for the nano_ATZ and ATZ tests, respectively. Hence, the controls were pooled (in each test) for the graphs and statistical analysis. Nano_ATZ induced a decrease in the number of adults and juveniles at 50 and 100 mg ATZ per kg although there was a high variation from the mean. For ATZ, there were no effects on survival and there was a dose-dependent decrease in the number of juveniles (significant from 100 mg ATZ per kg). For Gesaprim, there were no significant effects on survival or reproduction up to 400 mg kg^{-1}.

Full life cycle test (FLCt)

The results on hatching (11 d) and adults' survival and reproduction (number of juveniles) (46 d), as determined by the FCLt, are shown in Fig. 4 and the ECx calculated are shown in Table 2. As for the ERT, there were no significant

Table 2 Summary of the effect concentrations (ECx with 95% confidence intervals – CI), expressed as mg ATZ per kg soil, for *Enchytraeus crypticus* exposed to nano-encapsulated atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and Gesaprim in the LUFA 2.2 soil. The models used are Threshold sigmoid 2 or 3 parameters (Thres2P or 3P) or Logistic 2 parameters (Log2P). *S*: slope; y0: top point; n.e.: no effect; n.d.: not determined

Test substance	Test	Endpoint	EC_{10} (95% CI)	EC_{50} (95% CI)	EC_{90} (95% CI)	Model (parameters)
nano_ATZ	AVOID	Avoidance	n.e.	n.e.	n.e.	—
	ERT	Survival	29 (−220–277)	118 (68–168)	173 (10–336)	Thres2P (*S*: 6.2×10^{-3}; y0: 102)
		Reprod.	34 (−133–200)	114 (73–156)	195 (−6–396)	Log3P (*S*: 6.9×10^{-3}; y0: 101)
	FLCt	Hatching	153 (n.d.)	218 (n.d.)	259 (n.d.)	Thres2P (*S*: 8.5 10^{-3}; y0: 93)
		Survival	n.e.	n.e.	n.e.	—
		Reprod.	179 (n.d.)	276 (n.d.)	337 (n.d.)	Thres2P (*S*: 5.7×10^{-3}; y0: 97)
ATZ	AVOID	Avoidance	14 (−14–43)	101 (78–125)	156 (108–203)	Thres3P (*S*: 6.3×10^{-3}; y0: 110)
	ERT	Survival	n.e.	n.e.	n.e.	—
		Reprod.	11 (−33–54)	161 (130–191)	310 (237–383)	Log2P (*S*: 3.7×10^{-3}; y0: 104)
	FLCt	Hatching	122 (36–209)	208 (176–239)	293 (178–409)	Log2P (*S*: 6.4×10^{-3}; y0: 98)
		Survival	125 (62–188)	252 (186–319)	380 (210–551)	Log2P (*S*: 4.3×10^{-3}; y0: 97)
		Reprod.	95 (36–154)	236 (186–258)	376 (248–505)	Log2P (*S*: 3.9×10^{-3}; y0: 91)
Gesaprim	AVOID	Avoidance	11 (−1–122)	148 (61–357)	2012 (134–30 191)	Log2P (*S*: 0.48; y0: 82)
	ERT	Survival	n.e.	n.e.	n.e.	—
		Reprod.	n.e.	n.e.	n.e.	—
	FLCt	Hatching	n.d.	n.d.	n.d.	—
		Survival	378 (−5579–6334)	n.d.	n.d.	Log2P (*S*: 3.6×10^{-3}; y0: 89)
		Reprod.	206 (−10–421)	436 (304–561)	659 (298–1021)	Log2P (*S*: 2.4×10^{-3}; y0: 112)

differences between the controls of each test, thus the controls were pooled.

In terms of hatching, nano_ATZ and ATZ caused similar effects (EC_{50} = 218 mg nano_ATZ per kg and EC_{50} = 208 mg ATZ per kg), with a significant reduction at 200 mg ATZ per kg. For Gesaprim, there was a higher variability in the response, with the highest impact occurring at 100 mg kg^{-1}.

In terms of survival and reproduction, nano_ATZ caused no effects on adults' survival, while there was a reduction in the number of juveniles (EC_{50} = 276 mg ATZ per kg). ATZ caused a significant reduction in the number of adults and juveniles at 200 mg ATZ per kg, with a similar dose response curve: the LC_{50} and EC_{50} were 252 and 236 mg ATZ per kg, respectively. For Gesaprim, there was a reduction in the number of adults and juveniles above 200 mg ATZ per kg.

The organisms' length measurements (11 day juveniles and 46 day adults) showed that neither nano_ATZ nor ATZ affects the length, whereas Gesaprim caused a significant length increase in adults exposed to 400 mg ATZ per kg (ESI,† Fig. S1).

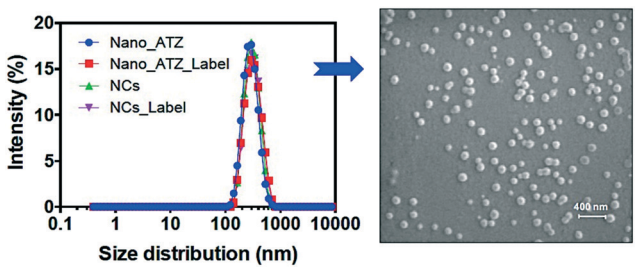

Fig. 1 Size distribution (intensity, %) of the nanoformulations by DLS: polymeric nanocapsules containing ATZ (●), labelled polymeric nanocapsules containing ATZ (■), polymeric nanocapsules (▲) and labelled polymeric nanocapsules (▼). Scanning electron microscopy of the nano_ATZ formulation with 50 000× magnification.

Uptake traceability assessment characterisation

The fluorescence was too low to be detected and no differences between the control and exposed were observed in any of the life stages (ESI,† Fig. S2).

Discussion

Material characterization showed that the nano_ATZ falls partly outside the nanomaterial range. This is the case for many studies dealing with "NMs", but more and more scientists have called for additional attributes to define a NM,[17] *e.g.* including size and surface area. For instance, in the EU the definition includes already some flexibility, 50% of the particles should be within that size range, but a fixed definition is not settled. Further, the EMA has also highlighted that materials below 1000 nm should be studied (see the EMA website). A recent editorial[18] further highlights that size measurements also vary depending on the method used (our study showed precisely the common differences between DLS (Table 1) and SEM (Fig. 1)). Most nanopesticides (usually larger than nanofertilizers) would not fit within the 100 nm size distribution definition, yet, some of the NM related properties remain and that should still require evaluation under the guidance for risk assessment of NMs applied to food and agriculture, as published by the European Food Safety Authority on 4 July 2018.[18]

In terms of avoidance behaviour, Gesaprim and pure atrazine (a.i.) caused similar avoidance response in *E. crypticus*, and the effects were in the same range as described for *E. albidus*.[19] Further, the estimated EC10 are in the range of the measured concentration of atrazine in soil (6 mg kg^{-1}, top 10 cm) as detected immediately after application in the field,[14] hence environmentally relevant and mimicking field applications. It is worth remembering that 6 mg kg^{-1} in the top 10 cm of soil indicate a much higher

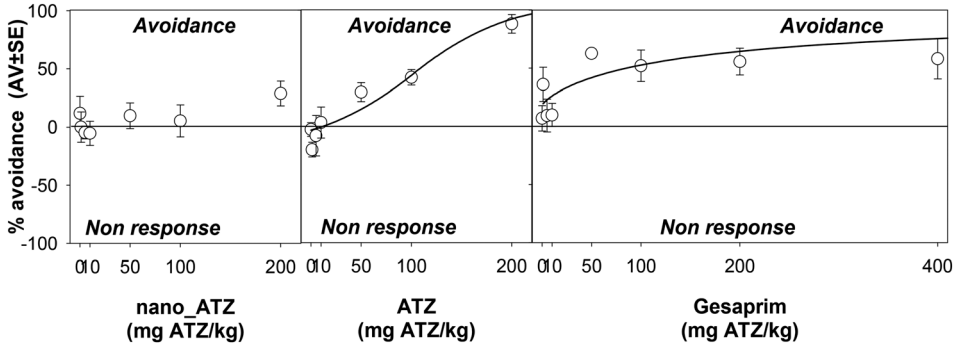

Fig. 2 Results of *Enchytraeus crypticus* avoidance response to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and Gesaprim, exposed for 48 h in the LUFA 2.2 soil. Lines represent the model fit to data.

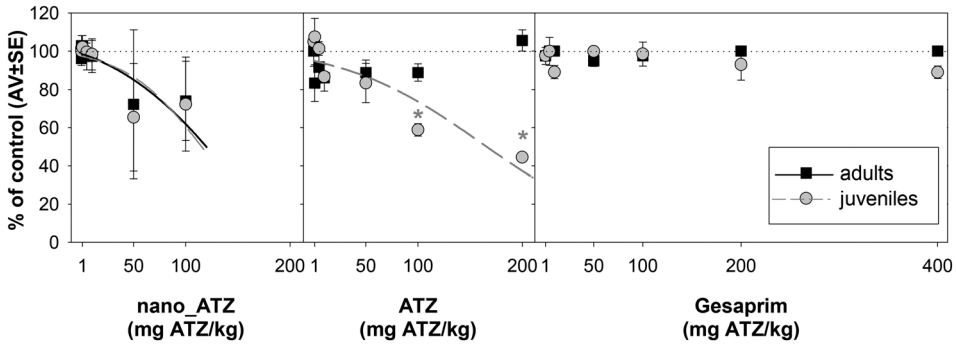

Fig. 3 Results of the standard enchytraeid reproduction test (ERT) in terms of survival and reproduction of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ) and pure atrazine, a.i. (ATZ), and Gesaprim, in the LUFA 2.2 soil. The results are presented as percentage of control (average ± standard error). $*p < 0.05$ (Dunnett's method).

concentration in the top 1–3 cm of soil which is also where more non-target organisms are present. These results suggest that the a.i., and not the inert substances of Gesaprim, is detected by the organisms and is responsible for the avoidance behaviour observed. The lack of avoidance to atrazine nanoformulation (nano_ATZ) can indicate that the nanoencapsulation reduced the chemical cue emission or, although less likely, that it affected the chemosensory capacity of the organisms. In addition, it can be related with the release kinetics of ATZ from the nanocapsules. Grillo *et al.*[5] showed that about 60% of ATZ was released from nano_ATZ, after 2 days in water, reaching a maximum of 70% after 5 days. In soil, this release kinetics is likely to be slower. This could mean that during the 2 day avoidance test the organisms were exposed to lower concentrations of ATZ (a.i.) than that in the ATZ test. This would explain our current results, for which the avoidance response at 200 mg ATZ per kg of nano_ATZ is similar to the response to 50 mg ATZ per kg of ATZ.

Based on the standard ERT, ATZ (a.i.) was more toxic to *E. crypticus* than Gesaprim. The higher efficacy (against the target organism) of the pure a.i. in comparison with the commercial formulations has been reported before, for instance, for the fungicide carbendazim.[20] Our current results indicate the same for the non-target organism *E. crypticus*, *i.e.*, higher toxicity of the pure a.i. The opposite has also been reported, *e.g.* Cavas[21] showed that Gesaprim induced genotoxicity on fish blood cells (*in vitro*) while atrazine (a.i.) was not genotoxic. ATZ toxicity was 80 times lower in *E. crypticus* (EC_{50} = 161 mg ATZ per kg) compared to that in *E. albidus* (EC_{50} = 2 mg ATZ per kg (ref. 22)). Differences between the sensitivity of the two species have been previously reported, for instance for cadmium and phenanthrene,[23] although not this high (about 5 to 6 times). The reproductive toxicity induced to *E. crypticus* by nano_ATZ and ATZ was similar.

The results of the FLCt showed that for ATZ (a.i.) and nano_ATZ the ECx were similar between hatching and reproduction, showing a good predictability between 11 and 46 day toxicity. This must mean that toxicity occurs at early stages of development. For ATZ, the effect on hatching persists over time, *i.e.*, reduction in hatching was irreversible, as observed by the reduced number of adults after 46 days. On the other hand, for nano_ATZ, hatching reduction was in fact a delayed development, as observed by the number of adults at day 46 (same as in controls). This was reported before, for other compounds such as $AgNO_3$ (ref. 24) or Ni-nanoparticles[25] for which the observed hatching reduction after 11 days was delayed, which was recovered at day 46. Despite the recovery in the number of adults, their reproductive output

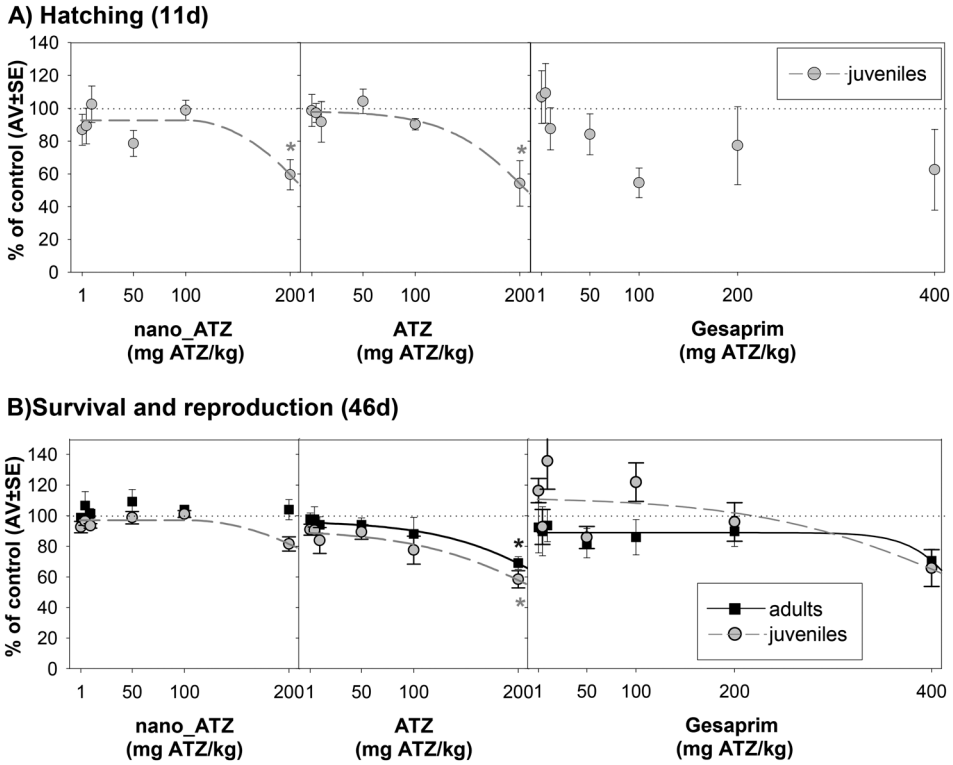

Fig. 4 Results of the full life cycle test (FCLt) in terms of A) hatching (11 days) and B) survival and reproduction (46 days) of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and Gesaprim, in the LUFA 2.2 soil. The results are presented as percentage of control (average ± standard error). *$p < 0.05$ (Dunnett's method).

was affected. This effect on reproductive output is in the same order of magnitude as hatching effects, at 11 days ($FLCt_{hatching}$ EC_{50} = $FLCt_{reproduction}$ EC_{50}) hence reflecting the toxicity to embryos/juveniles. For Gesaprim, the effects on hatching were more severe than the effects on survival and reproduction (less clear after 100 mg ATZ per kg due to higher variability at the higher concentrations) at day 46. This is also in line with embryos or recently hatched juveniles being more sensitive to the commercial formulation of atrazine.

Comparing the ERT and the FLCt, *i.e.* exposure from adults and from cocoons, for ATZ, the major differences were in terms of adults' survival (*i.e.*, no effects for the ERT, and LC_{50} = 252 mg ATZ per kg for the FLCt). This again confirms that, for ATZ, embryo or early development was the most affected life stage. For nano_ATZ, the ERT was more sensitive in terms of adults' survival (ERT LC_{50} = 118 mg ATZ per kg; FLCt LC_{50} > 200 mg ATZ per kg). This showed that for adults, the exposure to nano_ATZ in the ERT resulted in more toxicity than for adult organisms living in nano_ATZ spiked media in the FLCt. One possible explanation could be related with the higher uptake of nano_-ATZ by the adults exposed in the ERT. We were not able to confirm the uptake using fluorescent labelled nanocapsules containing atrazine since no fluorescence was detected (see Fig. S2†). The lack of fluorescence detection could be due to an inefficiency in the actual detection (*e.g.* due to high levels of organisms' auto-fluorescence) and/or after mixing with the soil media the fluorescence dilution factor is too high (the concentration may not be enough) for detection, hence it does not exclude uptake. As mentioned, up to 70% of ATZ is released from the nanocapsules within 5 days when in water,[5] indicating that, in the ERT, adult organisms would be exposed to a higher proportion of ATZ in the nanoform than the adults in the FLCt (which would be exposed to a higher proportion of released (*i.e.* free) ATZ). This could indicate that the higher toxicity (*i.e.* lower LC_{50}) observed in the ERT is the nano-related toxicity. A study by Jacques *et al.*[7] showed that the same nanoformulation of ATZ was highly toxic to *Caenorhabditis elegans* (inducing more than 50% mortality), however, the toxicity was caused, to a great extent, by the polymeric nanocapsule (NC) alone. Our results showed that the NCs alone did not affect *E. crypticus* in any of the endpoints, thus the effects reported here are due to nano_ATZ by different uptake mechanisms, by differentiated release rates of ATZ due to the nano-encapsulation, or by a combination of both.

In the FLCt, the organisms' reproductive capacity was affected almost at the same level for nano_ATZ and ATZ (a.i.). This effect on the reproductive output can be due to the endocrine disrupting action attributed to atrazine. For instance, adult zebrafish exposed to atrazine only during embryogenesis showed a reproductive dysfunction, this was associated to adverse effects induced to the neuroendocrine system.[26] Previous studies using the same nanoformulation

of ATZ showed lower toxicity in comparison to ATZ (a.i.) to human lymphocytes[5] and to the non-target maize plants.[6] For Gesaprim, the FLCt showed higher sensitivity than the ERT, as no effects were observed in the latter test. This indicated higher sensitivity of earlier life stages when organisms were exposed as cocoons, followed by some sort of resilience to the exposure, for instance by the activation of mechanisms of elimination and/or stress response. Adults, as exposed in the ERT, seem to handle exposure to Gesaprim better. The differences observed between the several forms of ATZ tested (nano, pure a.i. and commercial formulation) and in the sensitivity of the two life-stages (cocoons/embryos *versus* adults) suggest different mechanisms of toxicity. Further investigation should be done focusing on the understanding of those mechanisms to better predict the hazard of the (nano) formulations.

Overall, the results showed that nano_ATZ and pure ATZ were more toxic to *E. crypticus* than the commercial formulation, Gesaprim. Given that previous studies[27,28] showed that 10 times diluted nano_ATZ had the same herbicidal activity (against the target species *Brassica juncea*, *Bidens pilosa* and *Amaranthus viridis*) as the commercial formulation, this means that if nano_ATZ was applied as a weed control agent at 10 fold lower concentrations then the environmental risk could be reduced, but this requires an evaluation of the reduction in exposure concentration *versus* the higher toxicity of the nano-form.

Conclusions

This is among the first studies reporting the effects of a pesticide nanoformulation (in comparison to a commercial formulation and the respective a.i.) to a non-target soil invertebrate, *via* soil exposure. Overall, the results showed that the commercial formulation (Gesaprim) was the least toxic, and that nano_ATZ was not more toxic to *E. crypticus* than ATZ (a.i.) but that the hazard pattern may differ. Further investigation focusing on specific live stages (*e.g.* embryos) can elucidate the specific mechanisms of toxicity and contribute to improving the efficiency and safety of nanoformulations.

Conflicts of interest

There are no conflicts to declare.

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References

1 M. Kah and T. Hofmann, Nanopesticide research: current trends and future priorities, *Environ. Int.*, 2014, **63**, 224–235.
2 M. Nuruzzaman, M. M. Rahman, Y. Liu and R. Naidu, Nanoencapsulation, Nano-guard for Pesticides: A New Window for Safe Application, *J. Agric. Food Chem.*, 2016, **64**, 1447–1483.
3 M. Kah, R. S. Kookana, A. Gogos and T. D. Bucheli, A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues, *Nat. Nanotechnol.*, 2018, 1.
4 Y. Tong, Y. Wu, C. Zhao, Y. Xu, J. Lu, S. Xiang, F. Zong and X. Wu, Polymeric Nanoparticles as a Metolachlor Carrier: Water-Based Formulation for Hydrophobic Pesticides and Absorption by Plants, *J. Agric. Food Chem.*, 2017, **65**, 7371–7378.
5 R. Grillo, N. Z. P. dos Santos, C. R. Maruyama, A. H. Rosa, R. de Lima and L. F. Fraceto, Poly(ε-caprolactone) nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation, *J. Hazard. Mater.*, 2012, **231–232**, 1–9.
6 H. C. Oliveira, R. Stolf-Moreira, C. B. R. Martinez, G. F. M. Sousa, R. Grillo, M. B. de Jesus and L. F. Fraceto, Evaluation of the side effects of poly(epsilon-caprolactone) nanocapsules containing atrazine toward maize plants, *Front. Chem.*, 2015, **3**, 61.
7 M. T. Jacques, J. L. Oliveira, E. V. R. Campos, L. F. Fraceto and D. S. Ávila, Safety assessment of nanopesticides using the roundworm Caenorhabditis elegans, *Ecotoxicol. Environ. Saf.*, 2017, **139**, 245–253.
8 OECD, *No. 220. Enchytraeid Reproduction Test*, 2016.
9 R. C. Bicho, S. I. L. Gomes, A. M. V. M. Soares and M. J. B. Amorim, Non-avoidance behaviour in enchytraeids to boric acid is related to the GABAergic mechanism, *Environ. Sci. Pollut. Res.*, 2015, **22**, 6898–6903.
10 R. C. Bicho, F. C. F. Santos, M. F. M. Gonçalves, A. M. V. M. Soares and M. J. B. Amorim, Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*, *Ecotoxicology*, 2015, **24**, 1053–1063.
11 S. I. L. Gomes, C. P. Roca, N. Pegoraro, T. Trindade, J. J. Scott-Fordsmand and M. J. B. Amorim, High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, CuNO 3, and Cu salt aged): transcriptomics in *Enchytraeus crypticus*, *Nanotoxicology*, 2018, **12**, 325–340.
12 V. L. Maria, D. Licha, C. Ranninger, J. J. Scott-Fordsmand, C. G. Huber and M. J. B. Amorim, The *Enchytraeus crypticus* stress metabolome – CuO NM case study, *Nanotoxicology*, 2018, **12**, 766–780.
13 V. L. Maria, D. Licha, J. J. Scott-Fordsmand, C. G. Huber and M. J. B. Amorim, The Proteome of *Enchytraeus crypticus* - Exposure to CuO Nanomaterial and $CuCl_2$-in Pursue of a Mechanistic Interpretation, *Proteomics*, 2018, **18**, 1800091.

14 J. Gan, R. L. Becker, W. C. Koskinen and D. D. Buhler, Degradation of Atrazine in Two Soils as a Function of Concentration, *J. Environ. Qual.*, 1996, **25**, 1064.
15 M. Dehghani, S. Nasseri, S. A. Amin and Z. Zamanian, Assessment of Atrazine Distribution in Shiraz Soils, South of Iran, *Pak. J. Biol. Sci.*, 2010, **13**, 66–72.
16 ISO, No 17512-2, *Soil quality - avoidance test for determining the quality of soils and effects of chemicals on behaviour - part 2: test with collembolans (Folsomia candida)*, 2008.
17 A. D. Maynard, Don't define nanomaterials, *Nature*, 2011, **475**, 31–31.
18 Editorial, An opportunity not to be spoiled, *Nat. Nanotechnol.*, 2018, **13**, 619–619.
19 M. J. B. Amorim, S. Novais, J. Rombke and A. M. V. M. Soares, Enchytraeus albidus (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances, *Environ. Int.*, 2008, **34**, 363–371.
20 Sandhya, S. Kumar, D. Kumar and N. Dilbaghi, Preparation, characterization, and bio-efficacy evaluation of controlled release carbendazim-loaded polymeric nanoparticles, *Environ. Sci. Pollut. Res.*, 2017, **24**, 926–937.
21 T. Cavas, In vivo genotoxicity evaluation of atrazine and atrazine–based herbicide on fish Carassius auratus using the micronucleus test and the comet assay, *Food Chem. Toxicol.*, 2011, **49**, 1431–1435.
22 S. C. Novais, A. M. V. M. Soares and M. J. B. Amorim, Can avoidance in Enchytraeus albidus be used as a screening parameter for pesticides testing?, *Chemosphere*, 2010, **79**, 233–237.
23 M. P. Castro-Ferreira, D. Roelofs, C. A. M. van Gestel, R. A. Verweij, A. M. V. M. Soares and M. J. B. Amorim, *Enchytraeus crypticus* as model species in soil ecotoxicology, *Chemosphere*, 2012, **87**, 1222–1227.
24 R. C. Bicho, T. Ribeiro, N. P. Rodrigues, J. J. Scott-Fordsmand and M. J. B. Amorim, Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*, *J. Hazard. Mater.*, 2016, **318**, 608–614.
25 F. C. F. Santos, S. I. L. Gomes, J. J. Scott-Fordsmand and M. J. B. Amorim, Hazard assessment of nickel nanoparticles in soil-The use of a full life cycle test with *Enchytraeus crypticus*, *Environ. Toxicol. Chem.*, 2017, **36**, 2934–2941.
26 S. E. Wirbisky, G. J. Weber, M. S. Sepúlveda, T.-L. Lin, A. S. Jannasch and J. L. Freeman, An embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and morphological alterations in their offspring, *Sci. Rep.*, 2016, **6**, 21337.
27 L. Wang, T. Zhang, P. Li, W. Huang, J. Tang, P. Wang, J. Liu, Q. Yuan, R. Bai, B. Li, K. Zhang, Y. Zhao and C. Chen, Use of Synchrotron Radiation-Analytical Techniques To Reveal Chemical Origin of Silver-Nanoparticle Cytotoxicity, *ACS Nano*, 2015, **9**, 6532–6547.
28 G. F. M. Sousa, D. G. Gomes, E. V. R. Campos, J. L. Oliveira, L. F. Fraceto, R. Stolf-Moreira and H. C. Oliveira, Post-Emergence Herbicidal Activity of Nanoatrazine Against Susceptible Weeds, *Front. Environ. Sci.*, 2018, **6**, 12.

