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

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Answers to reviewers comments

Comments to the Author
The revisions were acceptable. Only a few minor grammatical errors to fix:

Line 98 "...6 mg atrazine/kg soil immediately..."
Answer: Done
Line 111-112 "...suspension was stirred for 10 min...evaporator to a final concentration of..."
Answer: Done
Line 124-125 "The concentrations and size distribution of the nanocapsules…"
Answer: Done
Line 263 "preparation, a monomodal" (the current 'a' is capitalized)
Answer: It is correct, it is a sentence start.
Line 374 "...website). A recent..." (the period was forgotten)
Answer: Done
Line 384-385 "...detected immediately after application..."
Answer: Done

Environmental Significance statement

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 pesticides 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 nanoformulation and the free atrazine. This illustrates the need for an evaluation of benefits (targets) versus risks (non-target).

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

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Abstract

The use of nanotechnology in the agrochemical sector aims to increase pesticides 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 the nano_ATZ. While all tested atrazine forms affected hatching success (11d, early development stage), 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), followed by the nano_ATZ (EC10 ca. 180 mg/kg) and atrazine (a.i.) (EC10 ca. 100 mg/kg). 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.

Keywords: nanopesticide; nanoencapsulation; avoidance; full life cycle; enchytraeids

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 in the nano-range size) 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 has 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, the 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 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 to 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 the 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 less comparing effects to 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 exposure 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 part 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 days enchytraeid reproduction test (ERT) to assess survival and reproduction, effects were assessed in terms of avoidance behaviour (2 days), cocoons 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/kg soil) and effects level (ECx) observed (see later), are within relevant field concentrations of atrazine (e.g. measurements detected ca. 6 mg atrazine/kg soil–~~when~~ 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/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 emollient) (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 the volume of 10 mL with the aid of a rotary evaporator~~, and atrazine at the~~ to a final concentration of 1 mg atrazine/mL. Additionally, labelled-polymeric nanocapsules were synthesized to trace 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.

Nanoparticles characterization

The 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. ~~To determine~~ The concentrations and size distribution of the nanocapsules containing

atrazine ~~was~~ were analyzed using the nanoparticle tracking analysis (NTA) technique. Data were collected through 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 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 - 4.0 and 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 $NaHCO_3$, at controlled conditions of temperature (19±1ºC) and photoperiod (16:8 hours light:dark). Cultures were fed on 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/100g, maximum water holding capacity (maxWHC) = 43.3 %, and 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, it is the a.i. of gesaprim and is further referred as ATZ. Polymeric nanocapsules containing atrazine (further referred as nano_ATZ) and polymeric capsules alone, to serve as control (further referred as NCs), were prepared as described above. The tested concentrations for gesaprim were 0-1-5-10-50-100-200-400 mg ATZ/kg soil dry weight (DW), and 0-1-5-10-50-100-200 mg ATZ/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 test start.

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 under the 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. Test started immediately thereafter.

For the 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. NCs controls (containing the polymeric nanocapsules without ATZ) were prepared using NCs (aqueous) dispersions. Soil was allowed to equilibrate for 1 day prior test start.

Test procedures

Avoidance tests. The avoidance tests were performed following the earthworm avoidance test guideline [16] using *E. crypticus* with adaptations as described in [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 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 ATZ test, the control for each comparison was the solvent control; an additional solvent control versus moist LUFA 2.2 soil was performed to assess the possible effects of acetone. For the nano_ATZ test, each test condition was performed versus the respective NCs control (e.g., for the concentration 50 mg ATZ/kg of nano_ATZ, the control was the NCs suspension at the same dilution); an additional NCs stock suspension versus moist LUFA 2.2 soil 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 [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 photoperiod of 16:8 h (light: dark). During the test duration, food (12 mg) and water content (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: NCs control, equivalent to the concentration 200 mg ATZ/kg for the nano_ATZ test). At the test end, the organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction assessed.

Full life cycle tests (hatching, growth, survival and reproduction). A reduced version of the full life cycle test (FLCt), as described in 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 days 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 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 ATZ test; 3: NCs controls, equivalent to the concentrations of 50 and 200 mg ATZ/ 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, 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/kg of labelled_nano_ATZ, from the cocoon stage (1-2 days 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 fluoresce lost). 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). Samples were collected at 7, 13, 25 and 46 days, under a stereomicroscope. The cocoons/organisms were washed in 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: $NR=((C-T)/N)\times100$, where C is the number of organisms observed in the control soil, T is the number of organisms observed in 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 NCs controls) were compared using t-test (for ATZ test) or One-Way Analysis of Variance (ANOVA) for NCs+ATZ test, at a significance level of 0.05. As there were no significant differences between controls, they were pooled prior 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 the various endpoints, modelling data to logistic or threshold sigmoid 2 or 3 parameters regression models, as indicated in Table 2, using the Toxicity Relationship Analysis Program (TRAP 1.30) software. Avoidance data was 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.

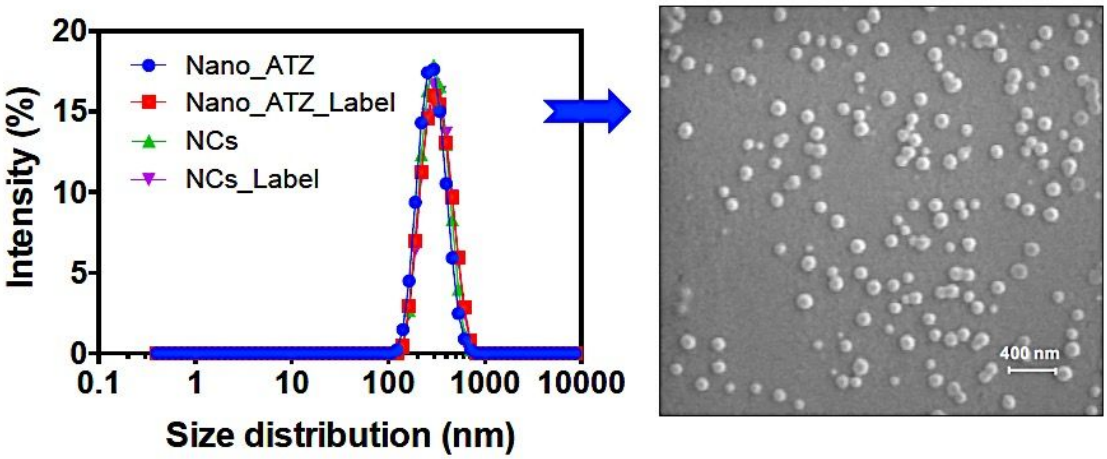

Fig. 1 Size distribution (intensity, %) of the nanoformulations by DLS: polymeric nanocapsules containing ATZ (●), labelled polymeric nanocapsules containing ATZ (□), C) polymeric nanocapsules (▲) and D) labelled polymeric nanocapsules (▼). Scanning electron microscopy of the nano_ATZ formulation by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

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).

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/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

We also evaluated the mean diameter of nanocapsules containing ATZ (nano_ATZ) by DLS after serial dilutions (see Table S1, Supporting Information). 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 of 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).

Avoidance response

Results on avoidance response are shown in Figure 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.

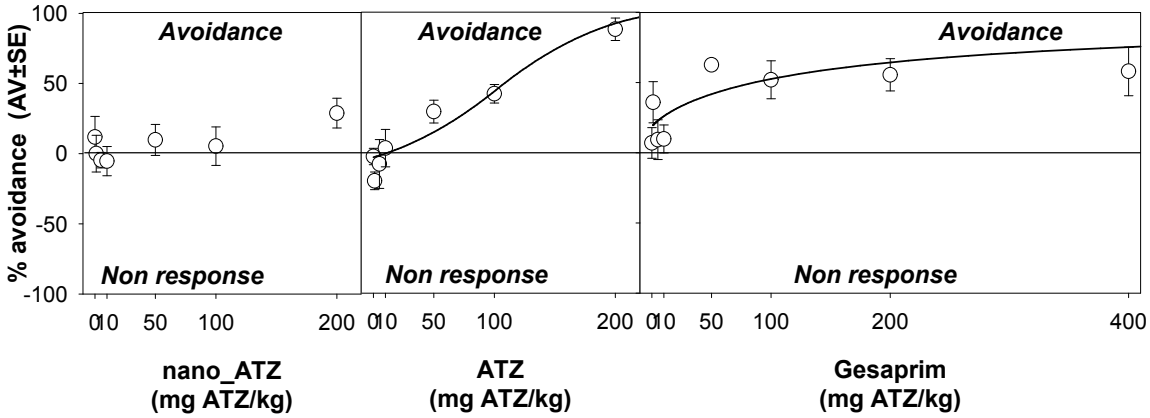

Fig. 2 Results of *Enchytraeus crypticus* avoidance response to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim, exposed for 48h in LUFA 2.2 soil. Lines represent the model fit to data.

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 significant (higher than 80% response) at 200 mg ATZ/kg. For gesaprim, there was more than 50% avoidance from 50 mg ATZ/kg; all the EC50s were estimated (Table 2).

Enchytraeid reproduction test (ERT)

Results on adults' survival and juveniles' production are shown in Fig. 3 and the ECx calculated, 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 control and control_NCs or 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/kg although there was a high variation from the mean. For ATZ, there were no effects on survival and a dose-dependent decrease in the number of juveniles (significant from 100 mg ATZ/kg). For gesaprim, there were no significant effects on survival or reproduction up to 400 mg/kg.

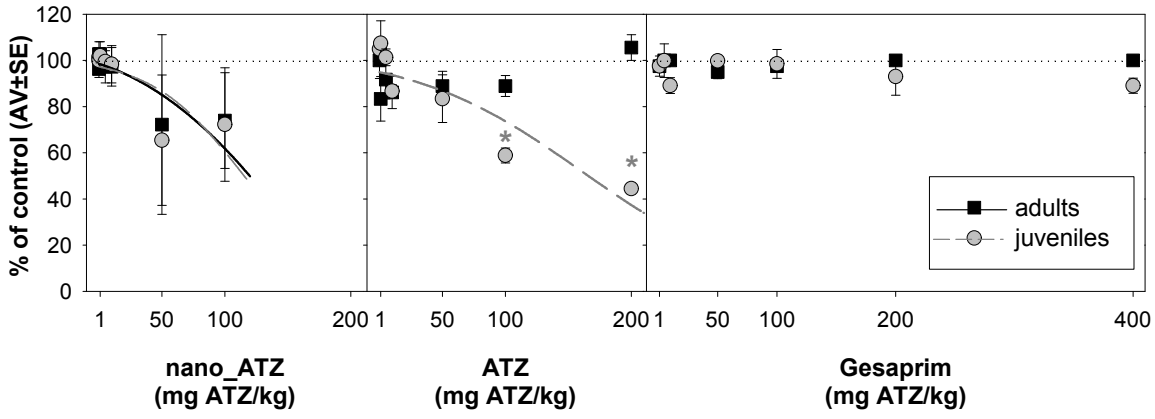

Fig. 3 Results of the standard Enchytraeid reproduction test (ERT) in terms of survival and reproduction of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ) and pure atrazine, a.i. (ATZ), and gesaprim, in LUFA 2.2 soil. Results are presented as percentage of control (average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

Full life cycle test (FLCt)

Results on hatching (11d) and adults' survival and reproduction (number of juveniles) (46d), as determined by the FCLt are shown in Fig. 4 and the ECx calculated in Table 2.

As for the ERT, there were no significant differences between the controls of each test, thus the controls were pooled.

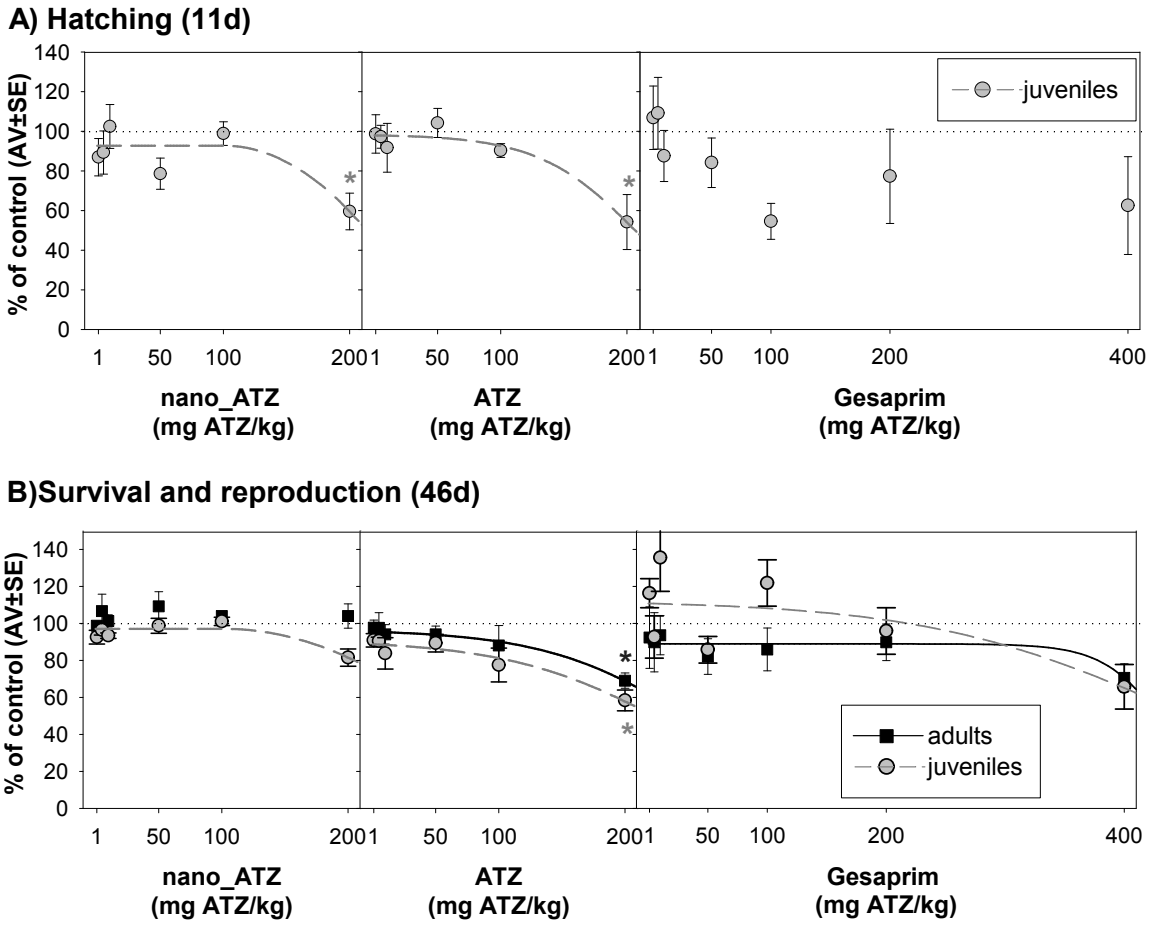

Fig. 4 Results of the Full Life Cycle test (FCLt) in terms of A) hatching (11 days) and B) survival and reproduction (46 days), of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim, in LUFA 2.2 soil. Results are presented as percentage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

In terms of hatching, nano_ATZ and ATZ caused similar effects (EC50=218 mg nano_ATZ/kg and EC50=208 mg ATZ/kg), with a significant reduction at 200 mg

ATZ/kg. For gesaprim, there was a higher variability in the response, with the highest impact occurring at 100mg/kg.

In terms of survival and reproduction, nano_ATZ caused no effects on adults´ survival, while there was a reduction in the number of juveniles (EC50 = 276 mg ATZ/kg). ATZ caused a significant reduction in the number of adults and juveniles at 200 mg ATZ/kg, with a similar dose response curve: the LC50 and EC50 were 252 and 236 mg ATZ/kg, respectively. For gesaprim, there was a reduction in the number of adults and juveniles above 200 mg ATZ/kg.

The organisms' length measurements (11 days' juveniles and 46 days' adults) showed that neither nano_ATZ or ATZ affect the length, whereas gesaprim caused a significant length increase in adults exposed to 400 mg ATZ/kg (SI, Fig S1).

Table 2 Summary of the effect concentrations (ECx with 95% confidence intervals - CI), expressed as mg ATZ/kg soil, for *Enchytraeus crypticus* exposed to nano-encapsulated atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim in LUFA 2.2 soil. The models used are Threshold sigmoid 2 or 3 parameters (Thres 2P or 3P) or Logistic 2 parameters (Log 2P). 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:6.2E-03; y0:102)
		Reprod.	34 (-133-200)	114 (73-156)	195 (-6-396)	Log3P (S:6.9E-03; y0:101)

		Hatching	153 (n.d.)	218 (n.d.)	259 (n.d.)	Thres2P (S:8.5E-03; y0: 93)
	FLCt	Survival	n.e.	n.e.	n.e.	-
		Reprod.	179 (n.d.)	276 (n.d.)	337 (n.d.)	Thres2P (S:5.7E-03 ; y0:97)
ATZ	AVOID	Avoidance	14 (-14-43)	101 (78-125)	156 (108-203)	Thres3P (S:6.3E-03 ; y0:110)
	ERT	Survival	n.e.	n.e.	n.e.	-
		Reprod.	11 (-33-54)	161 (130-191)	310 (237-383)	Log2P (S:3.7E-03 ; y0:104)
	FLCt	Hatching	122 (36-209)	208 (176-239)	293 (178-409)	Log2P (S:6.4E-03; y0:98)
		Survival	125 (62-188)	252 (186-319)	380 (210-551)	Log2P (S:4.3E-03; y0:97)
		Reprod.	95 (36-154)	236 (186-258)	376 (248-505)	Log2P (S:3.9E-03; y0:91)
Gesaprim	AVOID	Avoidance	11 (-1-122)	148 (61-357)	2012 (134-30191)	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:3.6E-03; y0:89)
		Reprod.	206 (-10-421)	436 (304-561)	659 (298-1021)	Log2P (S:2.4E-03; y0:112)

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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 (SI, Fig S2).

Discussion

Materials 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 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, EMA has also highlighted that material below 1000 nm should be studied (see 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 NMs 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, top 10 cm) as detected immediately after ~~the~~ application in the field [14], hence environmentally relevant and mimicking field applications. It is worth remembering that 6 mg/kg in top 10 cm, indicate a much higher concentration in the top 1-3 cm 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 cues 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 days avoidance test the organisms were exposed to lower concentrations of ATZ (a.i.) than in the ATZ test. This would explain our current results, for which the avoidance response at 200 mg ATZ/kg of nano_ATZ is similar to the response to 50 mg ATZ/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 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* (EC50 = 161 mg ATZ/kg) compared to *E. albidus* (EC50 = 2 mg ATZ/kg [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.

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 days' 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 $AgNO_3$ [24] or Ni-nanoparticles [25] for which the observed hatching reduction after 11 days was a delay, which was recovered by day 46. Despite the recovery in the number of adults, their reproductive output was affected, and this to the same order of magnitude as at day 11 hatching effects ($FLCt_{hatching}$ EC50 = $FLCt_{reproduction}$ EC50) 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 AZT/kg, due to higher variability in the higher concentrations), at 46d. 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 ERT, and LC50 = 252 mg ATZ/kg for 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 LC50=118 mg ATZ/kg; FLCt LC50 > 200 mg ATZ/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 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 LC50) observed in the ERT is 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 at the ATZ (a.i.), however, the toxicity was caused to a great extent, by the polymeric nanocapsule (NCs) 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 either by different uptake mechanisms, or by differentiated release rates of ATZ due to the nanoencapsulation, or a combination of both.

In the FLCt, organisms' reproductive capacity was affected almost at the same level for nano_ATZ and ATZ (a.i.). This effect on reproductive output can be due to the endocrine disrupting action attributed to atrazine. For instance, adult zebrafish exposed to atrazine only during embryogenesis showed 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, 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 from 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 from the ERT, seem to handle gesaprim exposure 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 becomes applied as weed control agent at 10 times 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 on specific mechanisms of toxicity and contribute to improve the efficiency and safety of nanoformulations.

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

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Abstract

The use of nanotechnology in the agrochemical sector aims to increase pesticides 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 the nano_ATZ. While all tested atrazine forms affected hatching success (11d, early development stage), 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), followed by the nano_ATZ (EC10 ca. 180 mg/kg) and atrazine (a.i.) (EC10 ca. 100 mg/kg). 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.

Keywords: nanopesticide; nanoencapsulation; avoidance; full life cycle; enchytraeids

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 in the nano-range size) 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 has 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, the 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 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 to 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 the 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 less comparing effects to 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 exposure 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 part 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 days enchytraeid reproduction test (ERT) to assess survival and reproduction, effects were assessed in terms of avoidance behaviour (2 days), cocoons 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/kg soil) and effects level (ECx) observed (see later), are within relevant field concentrations of atrazine (e.g. measurements detected ca. 6 mg atrazine/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/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 emollient) (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 the volume of 10 mL with the aid of a rotary evaporator to a final concentration of 1 mg atrazine/mL. Additionally, labelled-polymeric nanocapsules were synthesized to trace 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.

Nanoparticles characterization

The 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 through 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 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 - 4.0 and 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 $NaHCO_3$, at controlled conditions of temperature (19±1ºC) and photoperiod (16:8 hours light:dark). Cultures were fed on 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/100g, maximum water holding capacity (maxWHC) = 43.3 %, and 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, it is the a.i. of gesaprim and is further referred as ATZ. Polymeric nanocapsules containing atrazine (further referred as nano_ATZ) and polymeric capsules alone, to serve as control (further referred as NCs), were prepared as described above. The tested concentrations for gesaprim were 0-1-5-10-50-100-200-400 mg ATZ/kg soil dry weight (DW), and 0-1-5-10-50-100-200 mg ATZ/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 test start.

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 under the 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. Test started immediately thereafter.

For the 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. NCs controls (containing the polymeric nanocapsules without ATZ) were prepared using NCs (aqueous) dispersions. Soil was allowed to equilibrate for 1 day prior test start.

Test procedures

Avoidance tests. The avoidance tests were performed following the earthworm avoidance test guideline [16] using *E. crypticus* with adaptations as described in [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 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 ATZ test, the control for each comparison was the solvent control; an additional solvent control versus moist LUFA 2.2 soil was performed to assess the possible effects of acetone. For the nano_ATZ test, each test condition was performed versus the respective NCs control (e.g., for the concentration 50 mg ATZ/kg of nano_ATZ, the control was the NCs suspension at the same dilution); an additional NCs stock suspension versus moist LUFA 2.2 soil 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 [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 photoperiod of 16:8 h (light: dark). During the test duration, food (12 mg) and water content (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: NCs control, equivalent to the concentration 200 mg ATZ/kg for the nano_ATZ test). At the test end, the organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction assessed.

Full life cycle tests (hatching, growth, survival and reproduction). A reduced version of the full life cycle test (FLCt), as described in 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 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 ATZ test; 3: NCs controls, equivalent to the concentrations of 50 and 200 mg ATZ/ 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, 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/kg 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 fluoresce lost). 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). Samples were collected at 7, 13, 25 and 46 d, under a stereomicroscope. The cocoons/organisms were washed in 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: $NR=((C-T)/N)\times100$, where C is the number of organisms observed in the control soil, T is the number of organisms observed in 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 NCs controls) were compared using t-test (for ATZ test) or One-Way Analysis of Variance (ANOVA) for NCs+ATZ test, at a significance level of 0.05. As there were no significant differences between controls, they were pooled prior 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 the various endpoints, modelling data to logistic or threshold sigmoid 2 or 3 parameters regression models, as indicated in Table 2, using the Toxicity Relationship Analysis Program (TRAP 1.30) software. Avoidance data was 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.

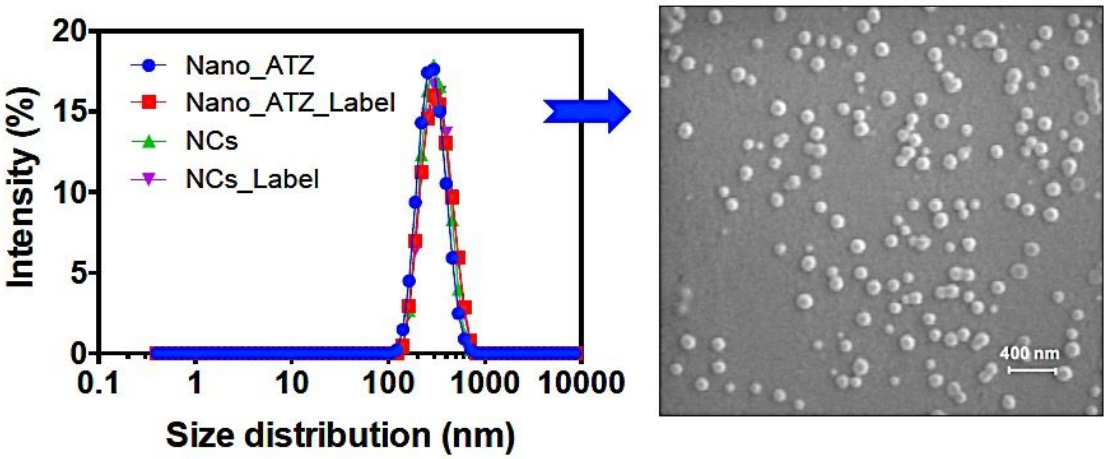

Fig. 1 Size distribution (intensity, %) of the nanoformulations by DLS: polymeric nanocapsules containing ATZ (●), labelled polymeric nanocapsules containing ATZ (□), C) polymeric nanocapsules (▲) and D) labelled polymeric nanocapsules (▼). Scanning electron microscopy of the nano_ATZ formulation by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

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).

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/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

We also evaluated the mean diameter of nanocapsules containing ATZ (nano_ATZ) by DLS after serial dilutions (see Table S1, Supporting Information). 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 of 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).

Avoidance response

Results on avoidance response are shown in Figure 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.

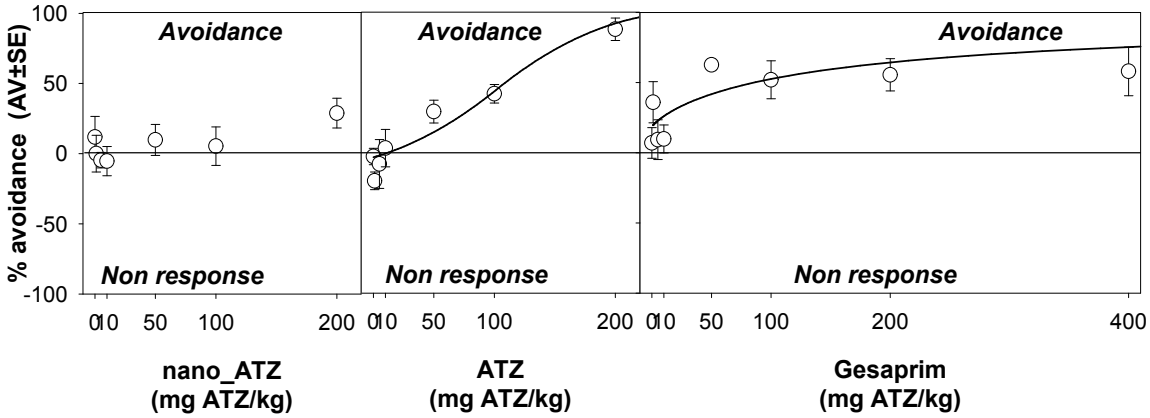

Fig. 2 Results of *Enchytraeus crypticus* avoidance response to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim, exposed for 48h in LUFA 2.2 soil. Lines represent the model fit to data.

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 significant (higher than 80% response) at 200 mg ATZ/kg. For gesaprim, there was more than 50% avoidance from 50 mg ATZ/kg; all the EC50s were estimated (Table 2).

Enchytraeid reproduction test (ERT)

Results on adults' survival and juveniles' production are shown in Fig. 3 and the ECx calculated, 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 control and control_NCs or 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/kg although there was a high variation from the mean. For ATZ, there were no effects on survival and a dose-dependent decrease in the number of juveniles (significant from 100 mg ATZ/kg). For gesaprim, there were no significant effects on survival or reproduction up to 400 mg/kg.

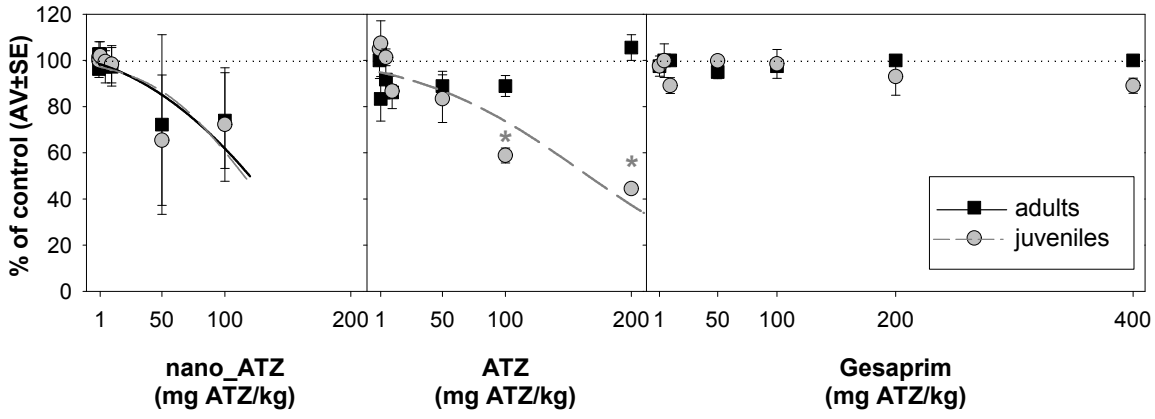

Fig. 3 Results of the standard Enchytraeid reproduction test (ERT) in terms of survival and reproduction of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ) and pure atrazine, a.i. (ATZ), and gesaprim, in LUFA 2.2 soil. Results are presented as percentage of control (average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

Full life cycle test (FLCt)

Results on hatching (11d) and adults' survival and reproduction (number of juveniles) (46 d), as determined by the FCLt are shown in Fig. 4 and the ECx calculated in Table

2. As for the ERT, there were no significant differences between the controls of each test, thus the controls were pooled.

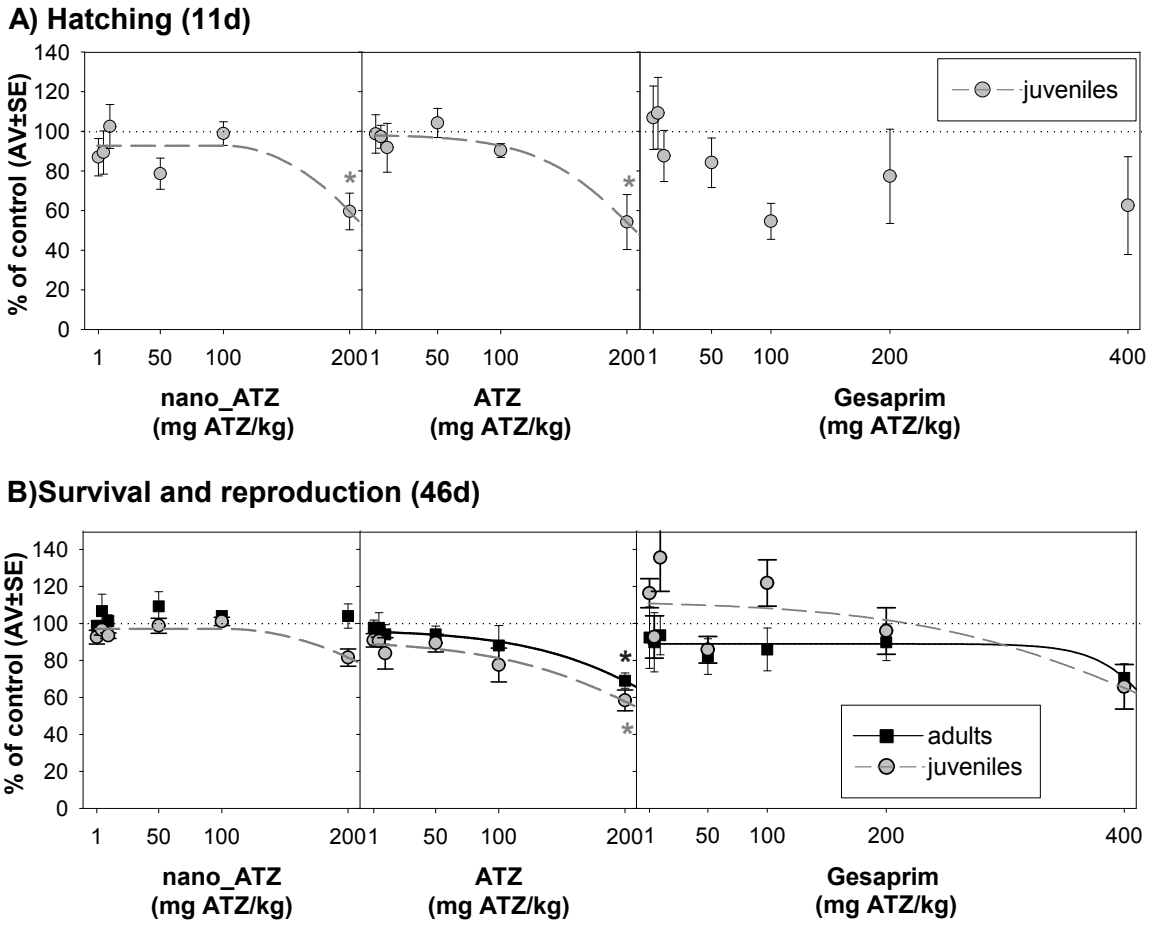

Fig. 4 Results of the Full Life Cycle test (FCLt) in terms of A) hatching (11 days) and B) survival and reproduction (46 days), of *Enchytraeus crypticus* exposed to nanocapsules containing atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim, in LUFA 2.2 soil. Results are presented as percentage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

In terms of hatching, nano_ATZ and ATZ caused similar effects (EC50=218 mg nano_ATZ/kg and EC50=208 mg ATZ/kg), with a significant reduction at 200 mg

ATZ/kg. For gesaprim, there was a higher variability in the response, with the highest impact occurring at 100mg/kg.

In terms of survival and reproduction, nano_ATZ caused no effects on adults´ survival, while there was a reduction in the number of juveniles (EC50 = 276 mg ATZ/kg). ATZ caused a significant reduction in the number of adults and juveniles at 200 mg ATZ/kg, with a similar dose response curve: the LC50 and EC50 were 252 and 236 mg ATZ/kg, respectively. For gesaprim, there was a reduction in the number of adults and juveniles above 200 mg ATZ/kg.

The organisms' length measurements (11 days' juveniles and 46 days' adults) showed that neither nano_ATZ or ATZ affect the length, whereas gesaprim caused a significant length increase in adults exposed to 400 mg ATZ/kg (SI, Fig S1).

Table 2 Summary of the effect concentrations (ECx with 95% confidence intervals - CI), expressed as mg ATZ/kg soil, for *Enchytraeus crypticus* exposed to nano-encapsulated atrazine (nano_ATZ), pure atrazine, a.i. (ATZ), and gesaprim in LUFA 2.2 soil. The models used are Threshold sigmoid 2 or 3 parameters (Thres 2P or 3P) or Logistic 2 parameters (Log 2P). 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:6.2E-03; y0:102)
		Reprod.	34 (-133-200)	114 (73-156)	195 (-6-396)	Log3P (S:6.9E-03; y0:101)

		Hatching	153 (n.d.)	218 (n.d.)	259 (n.d.)	Thres2P (S:8.5E-03; y0: 93)
	FLCt	Survival	n.e.	n.e.	n.e.	-
		Reprod.	179 (n.d.)	276 (n.d.)	337 (n.d.)	Thres2P (S:5.7E-03 ; y0:97)
ATZ	AVOID	Avoidance	14 (-14-43)	101 (78-125)	156 (108-203)	Thres3P (S:6.3E-03 ; y0:110)
	ERT	Survival	n.e.	n.e.	n.e.	-
		Reprod.	11 (-33-54)	161 (130-191)	310 (237-383)	Log2P (S:3.7E-03 ; y0:104)
	FLCt	Hatching	122 (36-209)	208 (176-239)	293 (178-409)	Log2P (S:6.4E-03; y0:98)
		Survival	125 (62-188)	252 (186-319)	380 (210-551)	Log2P (S:4.3E-03; y0:97)
		Reprod.	95 (36-154)	236 (186-258)	376 (248-505)	Log2P (S:3.9E-03; y0:91)
Gesaprim	AVOID	Avoidance	11 (-1-122)	148 (61-357)	2012 (134-30191)	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:3.6E-03; y0:89)
		Reprod.	206 (-10-421)	436 (304-561)	659 (298-1021)	Log2P (S:2.4E-03; y0:112)

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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 (SI, Fig S2).

Discussion

Materials 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 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, EMA has also highlighted that material below 1000 nm should be studied (see 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 NMs 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, 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 in top 10 cm, indicate a much higher concentration in the top 1-3 cm 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 cues 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 days avoidance test the organisms were exposed to lower concentrations of ATZ (a.i.) than in the ATZ test. This would explain our current results, for which the avoidance response at 200 mg ATZ/kg of nano_ATZ is similar to the response to 50 mg ATZ/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 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* (EC50 = 161 mg ATZ/kg) compared to *E. albidus* (EC50 = 2 mg ATZ/kg [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.

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 days' 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 $AgNO_3$ [24] or Ni-nanoparticles [25] for which the observed hatching reduction after 11 days was a delay, which was recovered by day 46. Despite the recovery in the number of adults, their reproductive output was affected, and this to the same order of magnitude as at day 11 hatching effects ($FLCt_{hatching}$ EC50 = $FLCt_{reproduction}$ EC50) 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 AZT/kg, due to higher variability in the higher concentrations), at 46d. 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 ERT, and LC50 = 252 mg ATZ/kg for 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 LC50=118 mg ATZ/kg; FLCt LC50 > 200 mg ATZ/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 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 LC50) observed in the ERT is 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 at the ATZ (a.i.), however, the toxicity was caused to a great extent, by the polymeric nanocapsule (NCs) 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 either by different uptake mechanisms, or by differentiated release rates of ATZ due to the nanoencapsulation, or a combination of both.

In the FLCt, organisms' reproductive capacity was affected almost at the same level for nano_ATZ and ATZ (a.i.). This effect on reproductive output can be due to the endocrine disrupting action attributed to atrazine. For instance, adult zebrafish exposed to atrazine only during embryogenesis showed 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, 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 from 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 from the ERT, seem to handle gesaprim exposure 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 becomes applied as weed control agent at 10 times 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 on specific mechanisms of toxicity and contribute to improve the efficiency and safety of nanoformulations.

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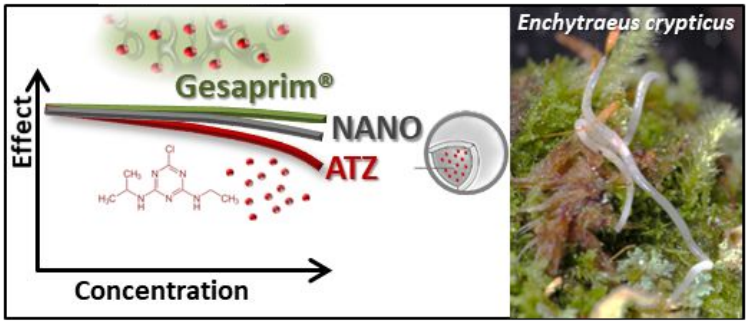

The commercial formulation of atrazine (Gesaprim) was the least toxic to *E. crypticus*, followed by nano_ATZ and and ATZ (a.i.).

Electronic Supplementary Information

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

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Table S1 Hydrodynamic diameter (Z average), using dynamic light scattering (DLS) of the polymeric nanocapsules (NCs) and nanocapsules containing atrazine (nano_ATZ): mean diameter is the average of 3 measurements (± standard deviation); polydispersity index (PDI).

material	equivalent soil concentration (mg ATZ/kg soil)	Diameter (nm)	PDI
NCs	1	221 ± 2	0.226
	5	219 ± 3	0.134
	10	224 ± 2	0.153
	50	219 ± 4	0.120
	100	223 ± 2	0.139
	200	219 ± 3	0.132
nano_ATZ	1	238 ± 3	0.237
	5	230 ± 1	0.155
	10	240 ± 1	0.162
	50	237 ± 1	0.173
	100	236 ± 1	0.171
	200	232 ± 3	0.172

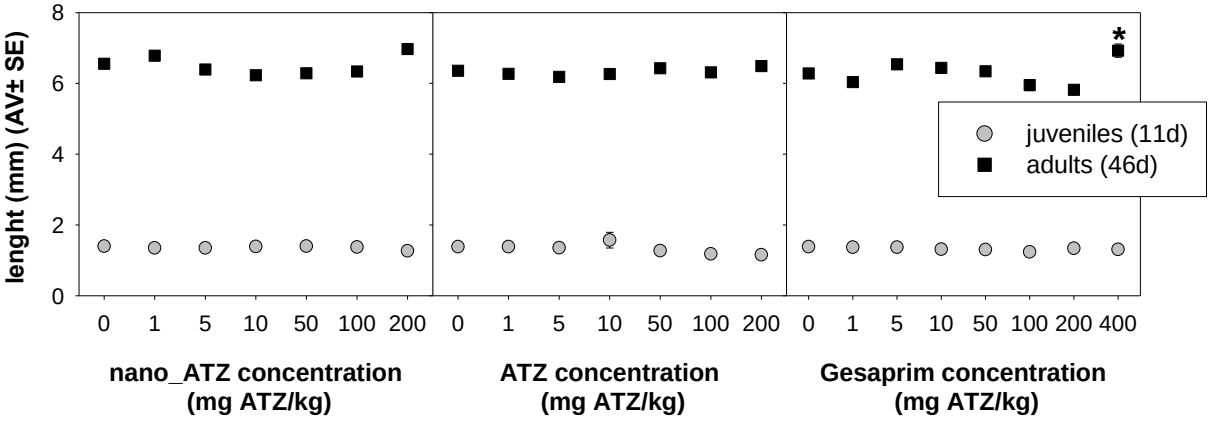

Fig. S1 Results of *Enchytraeus crypticus'* length after exposure to nanocapsules containing atrazine (nano_ATZ), pure atrazine (ATZ), and gesaprim, in LUFA 2.2 soil,

for 11 and 46 days. Results are presented as average ± standard error.). * $p<0.05$ (Dunn´s method).

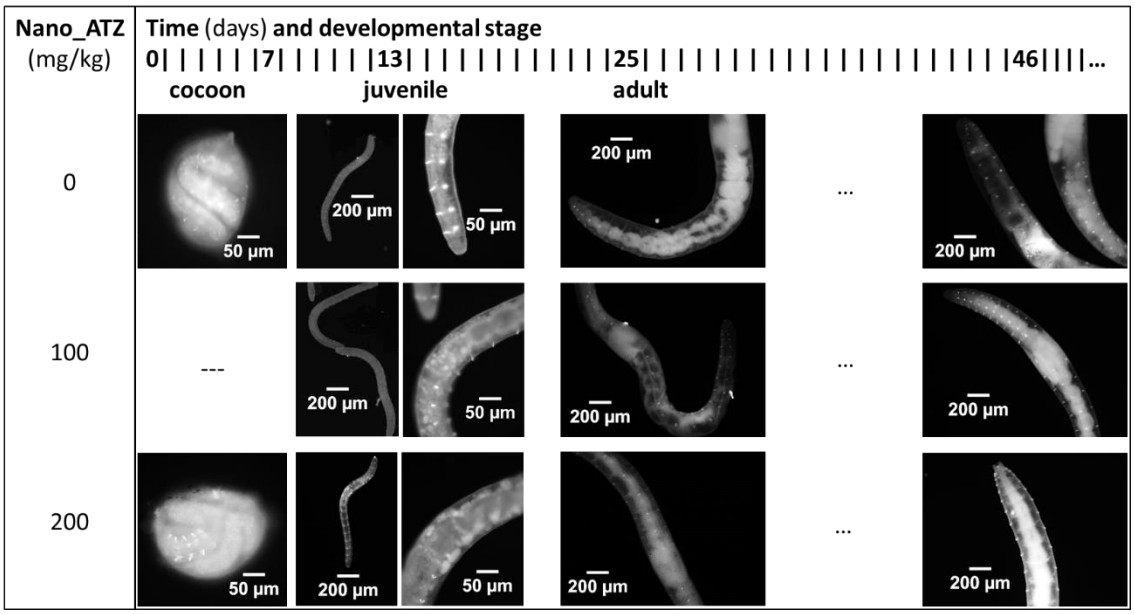

Fig. S2 Selected pictures from fluorescence microscope analysis of *Enchytraeus crypticus*, collected over time: cocoons (7 days), juveniles (13 days), and adults (25 and 46 days), when exposed to 0, 100 and 200 mg ATZ/kg of labelled nanocapsules containing atrazine (labelled_nano_ATZ) in LUFA 2.2 soil.