Eghbalinejad *et al.*
Environmental Sciences Europe (2024) 36:51
https://doi.org/10.1186/s12302-024-00879-9

Environmental Sciences Europe

RESEARCH

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Nano-enabled pesticides: a comprehensive toxicity assessment of tebuconazole nanoformulations with nematodes at single species and community level

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Abstract

There is an increasing imperative to explore safer alternatives for pesticides due to their indiscriminate use and consequential health impacts on the environment and humans. Nanoformulations of pesticides are being developed as potential alternatives due to their beneficial properties, including enhanced solubility, targeted delivery to the site of action, improved stability and efficacy and reduced non-target effects. Nevertheless, a comprehensive assessment is necessary for these emerging nanopesticides compared to existing formulations, aiming to ascertain whether their "nano" characteristics exacerbate toxicity for non-target organisms. This study investigated the toxicity of tebuconazole (TBZ) in different formulations, including nanoformulations (poly-ε-caprolactone [PCL] and nanostructured lipid carrier [NLC] loaded with TBZ), as well as a commercial formulation, on the reproduction of the nematode *Caenorhabditis elegans* in both aqueous and soil matrices. Additionally, the impact of the correspondent nanocarriers without TBZ on *C. elegans* was examined. In water, TBZ in the form of nano and commercial formulations exhibited higher toxicity on the nematodes' reproduction than the TBZ (a.s.) attributable to higher freely dissolved concentrations of TBZ, which resulted in a toxicity order, ranging from the most to the least toxic as follows: NLC-TBZ > PCL-TBZ > commercial formulation > TBZ (a.s.). For NLC-TBZ, the excess toxicity could be clearly explained by combined toxicity of TBZ (a.s.) and nanocarriers, with the effect addition of the separate single compounds matching the observed effects of the nanoformulation. For PCL-TBZ, effects were stronger than expected from the effect addition of TBZ (a.s.) and PCL nanocarriers, potentially due to enhanced bioavailability of encapsulated TBZ in the gut of the nematodes. In soil, NLC with and without loaded TBZ showed higher toxicity than other tested compounds, while PCL nanocarriers without TBZ did not exhibit negative effects on the reproduction of *C. elegans*. Microcosm experiment, where long-term effects on native soil nematode fauna were tested, confirmed that TBZ-nanoformulations act via combined toxic effects of TBZ and nanocarriers. These findings contribute valuable insights to understanding nanopesticides' ecotoxicity and underscore the need for harmonized regulatory assessments to evaluate these novel formulations adequately.

Keywords Combined toxicity, Nanotoxicity, *Caenorhabditis elegans*, Microcosm, Poly-ε-caprolactone, Tripalmitin

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Introduction

Pesticides have played a significant role in enhancing agricultural yields over the last decades, primarily by safeguarding seeds and crops against countless pests and diseases [1–3]. However, pesticides, by excessive application, have adverse environmental and human impacts through contamination of air, water, and soil, resulting in threats to biodiversity and sustainability [3–5]. In this regard, the European Commission recently adopted a strategy for sustainable pesticide use and aims to decrease the use and risks of chemical pesticides by 50% by 2030 [6]. Consequently, there is an urgent need for sustainable agricultural technologies. Nanotechnology has gained increasing attention in the field of agrochemicals over the current decade, with the potential to change the industry significantly [7, 8]. Nano-enabled pesticides (called nanopesticides), incorporating nanomaterials, enhance the effectiveness and reduce the environmental impacts of active substances (a.s.) through their nanoscale features. These innovations offer targeted delivery, improved efficacy and address the limitations of conventional pesticides, such as low solubility and rapid degradation [7, 9, 10]. This advancement represents a significant shift towards more efficient and environmentally friendly agricultural practices.

Different materials, such as polymers and lipids, are used to synthesize nanopesticides, with poly-ε-caprolactone (PCL) and nanostructured lipid carriers (NLCs) being notable examples due to their biodegradability, biocompatibility [11–13]. PCL, widely used in drug delivery [14, 15], has shown versatile degradation rates [16, 17]. It has been successfully utilized to make nanopesticides [13, 18–20], while NLCs, originally used in cosmetics and pharmaceuticals [21, 22], offer advantages in pesticide delivery [19, 20]. These nanocarriers (NCs) reduce the need for a.s., minimize environmental losses, and lower the ecological footprint, showcasing their potential to enhance agricultural sustainability [23, 24].

Nanopesticides, while promising for agriculture, require careful evaluation due to limited data on their environmental and toxicological impacts [25–27]. Their high surface-to-volume ratios and persistence [28] raise concerns about their transformation in the environment, potentially altering their fate and effects [7, 29]. Furthermore, their ability to enhance water solubility and bioavailability while protecting a.s. could extend environmental exposure, posing risks to non-target organisms [30, 31]. Therefore, conducting thorough research and risk assessments is essential to ensure that using nanopesticides in agriculture is safe and sustainable in the short and long term, particularly for non-target organisms.

The limited data on polymeric and lipid-based nanopesticides' effect on nematodes [32] stresses the pressing need to address this knowledge deficit in different exposure routes, such as water and soil for both short- and long-term effects. Nematodes are the most abundant and species-rich metazoans in soil and are essential in food webs [33, 34]. Moreover, nematodes are excellent indicators of chemical stress in soils and aquatic environments [35]. The nematode *Caenorhabditis elegans,* naturally occurring in microbe-rich habitats such as decaying plant material [36], is a standard test organism with lethal and sublethal toxicity endpoints to assess chemical toxicity in different environment compartments, such as soil, sediments, and water [37, 38]. This invertebrate species is used as a model organism due to its advantages, which fill the gap between in vitro and in vivo tests in addition to the simplicity, accuracy, repeatability, and low cost of the experiments [35, 39]. From an ecological perspective, chronic tests with sublethal parameter, such as reproduction, are preferred to acute tests that assess mortality [40, 41]. Moreover, laboratory multispecies test systems (small-scale microcosms) with native soil nematodes [42, 43] have been shown to be more sensitive to pesticide stress than single-species tests [44, 45].

Tebuconazole (TBZ) is an active substance in triazole fungicides widely used in agriculture to treat fungal diseases that act by inhibiting sterol biosynthesis in fungi (demethylation inhibitor) [46, 47]. It is the 8th most used fungicide in the European Union, with 1230 tonnes annually, a 1.1% share in fungicides used in the European Union [48]. It has low water solubility (36 mg/L at 25 °C) and 2.5–5 pKa) [49]. TBZ is recognized as a highly hazardous substance with broad-spectrum toxicity affecting cold-water, warm-water, and estuarine/marine organisms [50]. Its adverse effects include reproductive toxicity, liver tumors, metabolic abnormalities, and endocrine disruption [51–53]. Additionally, TBZ poses risks to birds and mammals [54]. Moreover, it is on the EU list of candidates for substitution [55, 56]. Therefore, the significant risks associated with TBZ demand enhanced application practices and the development of safer pest control alternatives to support safe and sustainable agriculture.

This study aimed to evaluate the reproductive toxicity of *Caenorhabditis elegans* exposed to two nanoformulations of tebuconazole (TBZ)—poly-ε-caprolactone (PCL-TBZ) and nanostructured lipid carriers (NLC-TBZ)—in aqueous and soil matrices. Our focus was to assess both the chemical toxicity of TBZ and the potential physical toxicity of the nanocarriers. We examined the effects of PCL-TBZ and NLC-TBZ, TBZ (a.s.), and nanocarriers without TBZ (NCs), as well as a commercially available TBZ formulation (FOLICUR®). The analysis of freely dissolved concentrations (only water exposure) allowed for comparing effects based on the bioavailable concentration of TBZ in all formulations.

Several hypotheses guided our research: (1) TBZ nanoformulations and commercial formulation are more toxic than TBZ (a.s.) due to additional formulation effects; (2) the toxicity of nanoformulations can be predicted by mixture toxicity models based on independent action; (3) combined toxic effects of TBZ and nanocarriers are also present in soil; (4) exposure to various TBZ formulations will alter the taxonomic composition of indigenous soil nematodes. This comprehensive approach aims to elucidate the environmental impact of TBZ nanoformulations and guide safer agricultural practices.

Materials and methods

Reagents and chemicals

The analytical standards of tebuconazole with a purity of > 98.0% (TBZ), poly-ε-caprolactone (PCL), polysorbate 80 (Tween 80), sorbitol monostearate surfactant (Span 60), polyvinyl alcohol (PVA), glycerol tripalmitate, triglycerides of capric and caprylic acids (in the form of Myritol 318), silica suspension (Ludox TM50®) and HPLC-grade acetonitrile (ACN) were purchased from Sigma Aldrich (Czech Republic). Acetone (≥ 99.5%) was purchased from Carl Roth GmbH, Karlsruhe, Germany. FOLICUR® (suspoemulsion; Bayer, 25 g TBZ/L) was purchased from a local supplier. Deionized water (dH_2O) was provided from a waters ultra-pure water system. The QuEChERS Extract bags (Agilent 5982-7650) were purchased from Agilent Technologies, USA. All other chemicals and solvents were analytical grade. Lufa St. 2.2 soil was provided by Landwirtschaftliche Forschungs- und Untersuchungsanstalt Speyer, Germany.

The preparation of nanoformulations and nanocarriers

The TBZ nanoformulations (NFs) were prepared following the methodologies outlined by Grillo et al. [13] for PCL-TBZ and Oliveira et al. [57] for NLC-TBZ (see Additional file 1: S1). The nanocarriers (NCs) were prepared using the same method, with the exception that TBZ was not added to the suspension. Physical and chemical properties of these NFs were rigorously analyzed, including hydrodynamic diameter, polydispersity index (PI), zeta (ζ) potential, and encapsulation efficiency of TBZ, using multi-angled dynamic light scattering technique (MADLS®, Zetasizer Ultra (Malvern Panalytical Ltd, UK). The ζ-potential measurements were performed on Zetasizer Ultra (Malvern Panalytical Ltd, UK) using the Electrophoretic Light Scattering (ELS) method. For the morphology of nanoparticles, Transmission Electron Microscopy (Philips 208 S Morgagni (FEI, Czech Republic)) was used. The total concentration of TBZ loaded in PCL-TBZ and NLC-TBZ and released TBZ from nanocarriers were measured by high-performance liquid chromatography–mass spectrometry (HPLC–MS/MS). Details are provided in Additional file 1: S1–S4. The detail on HPLC–MS/MS is provided by Lopez-Cabeza et al. [20].

Preparation of stock solutions and spiking procedure

An overview of all experiments with information on the respective exposure conditions can be found in Additional file 1: Table S1.

TBZ (a.s.) in water and soil

To prepare the chemical solutions of TBZ (a.s.) for the experiments in water (Exp 1) and soil (Exp 2), stock solutions were prepared in acetone. Based on the results of range-finding tests, a concentration series with ten concentrations (for water tests), six and three concentrations (for soil tests) were prepared. For water tests, acetone stock solutions were prepared in 200-fold concentrations to achieve a final solvent concentration of 0.5% in the exposure medium used within the test vessels as the concentration of acetone was as follows for the given tested concentration from lowest to the highest tested concentration: 1.04, 1.56, 2.34, 3.51, 5.27, 7.90, 11.9, 17.8, 26.7 and 40.0 mg/mL acetone. Spiking 3.98 mL of test medium with 20 µl of acetone stock solution (20 µl of acetone for the solvent control), aqueous, double-concentrated stock solutions, have been achieved. After 1:1 dilution with the food medium (bacterial suspension), the final test concentrations were achieved: 0 (solvent control), 5.2, 7.8, 11.7, 17.6, 26.3, 39.5, 59.3, 88.9, 133, and 200 mg/L. Despite the limited water solubility of TBZ (36 mg/L [49]), higher nominal concentrations were tested to account for potential higher exposure concentrations due to bacterial-bound TBZ.

For soil tests (Exp 2; see Additional file 1: Table S1), the following acetone stock solutions were prepared for TBZ (a.s.): 0.10, 0.17, 0.29, 0.49, 0.84, 1.42 (test 1) and 0.38, 0.75, 1.50 (test 2) mg/mL acetone. Spiking 20 g dry soil (Lufa standard soil St.2.2; sandy loam; 8.3% clay, 14.5% silt, 77.2% sand, 1.71% TOC, pH: 5.6, WHCmax: 44.8%) with 10 mL of acetone (acetone stock solution and pure acetone (solvent control), following final soil test concentrations have been achieved: 0 (solvent control), 60, 100, 173, 295, 501 and 852 (test 1) and 0 (solvent control), 188, 355 and 750 (test 2) mg/kg dry soil. After the complete evaporation of acetone, solvent concentrations in the soil were negligible (according to our previous test).

Based on the results of tests with TBZ (a.s.), concentration series for FOLICUR® (six nominal test concentrations: 5.2, 11.7, 26.3, 59.3, 133 and 200 mg/L), PCL-TBZ and NLC-TBZ (eight nominal test concentrations: 5.2, 7.8, 11.7, 17.6, 26.3, 38.5, 88.9, and 200 mg/L) were set up. To achieve maximally released TBZ (a.s.) in the medium from the TBZ NFs, the prepared stock solutions of all

compounds were slightly shaken in an incubator for 48 h at 20 °C. The concentrated solutions were mixed with food suspension (1:1) and kept in the incubator for 2 h at 20 °C to allow for equilibrium partitioning between water and bacteria. After that, the prepared solutions were distributed into final glass vials for the experiments ($n=4$).

For test 2, the soil was also spiked with FOLICUR®, PCL-TBZ and NCL-TBZ, and the respective NCs. As for TBZ (a.s.), nominal concentrations were 188, 355, and 750 (test 2) mg/kg dry soil. Here, dry soil was spiked with aqueous stock solutions and dried till complete evaporation of the water.

The NCs` solutions (without TBZ) were simultaneously prepared based on the equivalent volume used for their corresponding NFs (with TBZ), which resulted in varying number of particles and concentrations of other compounds (used in the preparation of nanoformulations) across treatments. However, due to the DLS limitations, the particle concentration used for mixture analysis and soil effect analysis (Figs. 1, 2) are calculated based on the measured particle concentration in stock suspensions (Additional file 1: Table S2).

Preparation of food medium

Bacterial suspensions (*Escherichia coli*. strain OP50) were prepared as food for *C. elegans* following standard procedures ISO 10872 [38]. Briefly, bacteria were grown in Luria–Bertani (LB) medium, centrifuged, and the pellet was washed and resuspended in M9-medium to achieve densities suitable for water and soil tests. The detailed preparation process, including growth conditions, centrifugation, compositions of LB and M9 media, and resuspension, is provided in Additional file 1: S6.

Chemical analysis

Water

Chemical analyses were conducted for all tested compounds including TBZ (a.s.), FOLICUR®, PCL-TBZ, and NLC-TBZ two times. Firstly, after incubating the solutions for 48 h, the chemical analysis was done as an aliquot of the solution was taken to measure TBZ concentration by HPLC–MS–MS ($n=3$). The last chemical analysis was at the end of the tests, after 146 h. PCL-TBZ and NLC-TBZ were analyzed for both total concentration (C_{tot}) and released concentration of TBZ (freely dissolved TBZ in media which is not associated with nanocarriers (C_{free})). The method for chemical analysis was the same used for the method explained in the chemical characterization of nanoformulations section (Additional file 1: S4). The chemical analysis of water samples was conducted separately from the toxicity tests due to infrastructure limitations. However, it is important to note that these analyses were meticulously performed to reflect the conditions of the toxicity tests accurately.

Soil (toxicity test and microcosm test)

Soil samples were analyzed for the total content of TBZ using the QuEChERS extraction method. This method has been used in different studies, demonstrating it is a fast, easy, and effective method to remove different pesticides from environmental samples using salt [31, 58, 59]. The extraction procedure was performed as follows: all samples were lyophilized for 48 h. Then, 0.1 g dw of spiked soil was shaken with 5 mL deionized water and 10 mL acetonitrile with metolachlor added as a surrogate standard (10 µL of 1000 µg/mL concentration per sample). Samples were further amended with 6.5 g of QuEChERS Extract bag (4 g MgSO4, 1 g NaCl, 0.5 g disodium citrate sesquihydrate, 1 g Na citrate). The mixture was shaken by a shaker (SPEX®SamplePrep) for 1 min and then centrifuged for 5 min/3000 rpm/−5 °C. Then acetonitrile layer (top layer) was transferred to clean 20 mL glass vials. The extract was later diluted ten times with 50% acetonitrile to align with the HPLC–MS/MS detection range of 1–100 ng/mL. An aliquot of 1 mL of acetonitrile was taken for analysis using HPLC–MS/MS. Details on the analysis are provided by López-Cabeza et al. [20].

Toxicity test with *Caenorhabditis elegans*

The toxicity test with *C. elegans* was carried out according to standard procedures (ISO 10872) [38]. To cultivate *C. elegans*, nematode growth medium (NGM) was used, which is described in detail in Additional file 1: S5. All agar plates were seeded with OP50, an uracil-requiring mutant of *Escherichia coli* that prevents overgrowth of the bacterial lawn [60] following standard procedures [61]. Stock culture plates containing dauer larvae of *C. elegans* were then stored at 20 °C in the dark. Three days before the start of a test, small pieces of a stock plate with dauer larvae were transferred to NGM plates with fresh bacterial lawn and incubated at 20 °C, resulting in freshly hatched juveniles (J1) that could be used for toxicity testing.

Nematodes were exposed to the test items in 10 mL glass vials. Six *C. elegans* (J1) were introduced in each replicate vial containing either spiked aqueous medium or soil (moistened to 80% WHC_{max}). The vials were kept in the incubator for 96 h at 20 °C. The experiment was stopped by heat-killing the nematodes in an oven (18–20 min at 80 °C) after adding Bengal rose solution to stain the nematode cuticle for better recovery. The vials were kept in the fridge till further analysis.

For soil tests, nematodes had to be separated from soil particles before reproduction could be analyzed. This was

done by density separation using a flotation/centrifugation technique with a silica suspension (diluted with water to 1.13 g/mL) [38]. For water tests, this step was not necessary.

To measure the reproduction of *C. elegans*, we counted the number of offspring (second-generation juveniles) using a stereo microscope at 40-fold magnification. In soil experiments, the offspring were counted in a silica suspension after being separated from the soil. In water experiments, the offspring were counted directly in the aquatic medium. The total number of offspring was then divided by the number of introduced test organisms ($n=6$), less the number of males. An effect was defined as inhibition of reproduction (% IR) compared to the control reproduction:

$$\%IR_A = 100 - \frac{\overline{x}_A}{\overline{x}_C} \times 100, \quad (1)$$

where $\overline{x}_{A,}$ and $\overline{x}_C$ are the mean values reproduction for sample A and control, respectively.

Microcosm test

Based on the results of the *C. elegans* toxicity tests, PCL-TBZ (NF with low carrier effects) and FOLICUR® were selected for assessing their toxicity on a native soil nematode fauna in small-scale (30 g soil) microcosms [42]. PCL nanocarrier (without TBZ) was tested as well. Due to the higher effort of the microcosm test compared to the single-species test, only one concentration of each compound was tested in four replicates in addition to a control treatment (only water). The test vessels were 50 mL plastic tubes which were filled with 30 g of spiked soil including the native nematode fauna. Fresh soil (freshly sampled soil (Lufa St. 2.2).) The soil was spiked by mixing a dried aliquot of the Lufa soil (10% = 12 g dry soil) with the respective aqueous stock suspension of the test item (PCL-TBZ: 8.3 mL of 2 g a.s./mL; FOLICUR®; 16.7 mL of 2.5 mg a.s./mL), drying the spiked soil aliquot and mixing it with 126 g of fresh soil. This resulted in a nominal soil concentration of 350 mg TBZ (a.s.)/kg dry soil. For chemical analysis at $t=0$, 4 g of soil was sampled from each treatment and kept frozen at −20 °C till further analysis (see section "Soil (toxicity test and microcosm test)"). From the unspiked soil, 30 g soil was also sampled for nematode community analysis to identify the nematode genus composition at $t=0$.

After an 8-week exposure period at 15 °C, microcosms were processed as follows: first, 1 g of soil from each microcosm was collected for chemical analysis. The samples from the four replicates of each treatment were combined, resulting in 4 g per treatment, and stored at −20 °C until analysis (see section "Soil (toxicity test and microcosm test)" for details). Secondly, the remaining soil was mixed with a silica suspension (explained in section "Toxicity test with *Caenorhabditis elegans*") to facilitate the separation of nematodes from the soil [42]. The mixture was centrifuged for 10 min at 800*g*, and the supernatant was filtered through a 10-µm gauze to remove the silica while retaining the nematodes, which were collected into a separate container. This flotation and centrifugation process was performed two times to ensure thorough extraction. The resulting nematode suspension, a combination of all extraction steps, was fixed with formaldehyde (final concentration of 4%), stained with Bengal Rose for enhanced visibility of the nematodes, and stored at room temperature for subsequent analysis. Using a stereo microscope (40-fold magnification), nematodes of each microcosm were counted (total nematode density). The first 140 individuals (124–153) were prepared for taxonomic identification. Nematode taxa were categorized into functional groups in terms of their feeding type [62] and life-history strategy [63] so that maturity indexes (MI; MI25) could be calculated to allow evaluation of soil health [33].

Data evaluation and statistical analysis

All the data were checked for normality and homogeneity in parametric analysis by GraphPad Prism 9.5.1 (GraphPad Software, USA). The Kolmogorov–Smirnov test was applied to assess normality before conducting further analyses. In cases where the assumptions of normality and homogeneity were not met, the data underwent log transformation.

We calculated the mean value of the measured TBZ concentrations for the exposure concentration of TBZ to the nematodes for both total concentration and free TBZ concentration at the beginning and end of the toxicity tests. Specifically, for the aquatic tests, these measurements were taken at $t=50$ h and $t=146$ h; for soil tests, at $t=0$ and $t=96$ h; and for microcosm studies, at $t=0$ and $t=8$ weeks. Concentration–response curves were fitted to data (% IR plotted versus exposure concentrations) using a sigmoidal logistic function (3 parameters, assuming a curve plateau at 100% inhibition).

One-way ANOVA (with Dunnett post hoc tests) was used for testing for significant differences between reproduction values in TBZ or carrier treatments and the respective negative controls to determine the No-Observed-Effect-Concentration (NOEC; concentration step below the lowest concentration that induced a significant effect).

The EC_{50} value for TBZ (a.s.) was statistically compared against the EC_{50} values of all TBZ formulations (FOLICUR®, PCL-TBZ, NLC-TBZ) with the *Z*-Test (Environment Canada, 2005) using Eq. 2 [64]:

$$Z = \frac{\log EC50_{TBZa.s.} - \log EC50_{TBZformulation}}{\sqrt{\sigma^2{}_{logEC50_{TBZa.s.}} + \sigma^2{}_{logEC50_{TBZformulation}}}}, \quad (2)$$

where σ represents the standard error.

Assuming independent action of TBZ and NCs, mixture toxicity was modeled by effect addition and plotted against "Toxic units (TU) mix ($=TU_{carrier}+TU_{TBZa.s.}$)" in order to get a dimensionless unit considering both types of toxic stress (for water tests).

Nematode genus composition was analyzed using multivariate statistics and ordination methods (non-metric multidimensional scaling [nMDS]; analysis of similarity [ANOSIM]) using PRIMER software (Version 6.1.5). Relative abundances of taxa were square-root transformed, and Bray–Curtis similarities were calculated. From this similarity matrix, a two-dimensional plot was generated so that the distance of samples is positively correlated with the dissimilarity in taxa composition.

Differences in univariate measures of the nematode community (taxa abundances, indexes) between the treatments and the control were statistically tested using one-way ANOVA (with Dunnett post hoc tests).

Results

Characterization of the nanoformulations and nanocarriers

The stock suspensions of the prepared NFs loaded with TBZ and the corresponding NCs (without TBZ) were chemically and physically characterized by measuring the total concentration of TBZ, hydrodynamic diameter (HDD), polydispersity index (PI), ζ potential, particle concentration and encapsulation efficiency (EE) (Additional file 1: Table S2). Total TBZ concentrations in the test medium were comparable to the expected nominal concentrations for PCL-TBZ (2000 µg/mL was expected, and 1660 µg/mL was measured) but slightly lower for NLC-TBZ (800 µg/mL was expected and 571 µg/mL was measured) (Additional file 1: Table S2). Physical characterization shows that PCL nanoparticles were smaller on average than NLC nanoparticles, with no significant size difference between NCs (with and without TBZ) ($p > 0.05$).

The chemical and physical characterizations of the stock suspensions used for the experiment in soil were similar to the stock suspension used for the experiment in water. However, the unloaded NLC was slightly different (Additional file 1: Table S2). The HDD for NLC nanoparticles was bigger than particles in other tested compounds at 328 ± 6 nm. The differences were not statistically significant ($p > 0.05$).

The relative differences in the physical characterization of the stock suspensions used for the microcosm study, the PCL, and PCL-TBZ (Additional file 1: Table S2) were not statistically significant ($p > 0.05$). The results suggest that the presence of TBZ does not significantly influence the stability and size properties of the PCL nanoparticles.

Based on the morphological characteristics of nanoformulations (stock) depicted in Additional file 1: Fig. S1, a remarkable alignment between transmission electron microscopy (TEM) analysis and dynamic light scattering (DLS) results is observed, validating the reliability of our observations. This correlation enhances confidence in the documented morphological and dimensional features of TBZ-loaded nanoparticles. Furthermore, the combined use of TEM and DLS contributes to a comprehensive understanding of the particulate system.

In general, the NLC samples demonstrate substantial polydispersity, showcasing a diverse range of particle sizes, including both larger and relatively smaller particles, as confirmed by TEM observations (Additional file 1: Fig. S1). The particle size distribution analysis reveals a prominent fraction of larger particles in the NLC samples (for distribution analysis, see Additional file 1: Figs. S2, S3), leading to a bigger size (average hydrodynamic diameter) compared to the more monodisperse PCL. It is noteworthy that the PI for all samples was relatively small, indicating an optimal level of uniformity despite the observed polydispersity in size (Additional file 1: Table S2, Additional file 1: Figs. S2, S3).

For tests with TBZ (a.s.) and NLC-TBZ in water, the measured TBZ freely dissolved concentrations (C_{free}) at the beginning of the toxicity test (T0) were considerably lower than the aimed nominal concentrations, even for values below the reported water solubility of TBZ (36 mg/L), showing 53–64% and 42–55% of the nominal concentrations for TBZ (a.s.) and NLC-TBZ, respectively (3.0–14.1 mg/L and 2.6–11.0 mg/L, respectively; Additional file 1: Table S3). For nominal concentrations above the water solubility, measured C_{free} expectedly showed only 23–56% and 12–32% of the nominal concentrations for TBZ (a.s.) and NLC-TBZ, respectively (21.8–45.4 mg/L and 12.8–24.8 mg/L, respectively; Additional file 1: Table S3). For tests with FOLICUR® and PCL-TBZ in water, the measured TBZ freely dissolved concentrations (C_{free}) at the beginning of the toxicity test (T0) corresponded well with the aimed nominal concentrations below the water solubility (74–113% of the nominal concentration). For nominal concentrations above the water solubility, measured C_{free} expectedly showed only 15–71% and 11–47% of the nominal concentrations for FOLICUR® and PCL-TBZ, respectively (27.5–31.4 mg/L and 18.4–22.2 mg/L, respectively; Additional file 1: Table S3). Within the test duration of 96 h, however, the C_{free} of TBZ did not change substantially in any of the tests in water (average absolute change: 1 ± 14.4%),

ensuring constant exposure concentrations for the nematodes (Additional file 1: Table S3).

For toxicity tests of TBZ (a.s.) and FOLICUR® in soil, measured total soil concentrations at the beginning of the test (T0) matched well the aimed nominal concentrations, showing 91–151% and 78–159%, respectively, of the nominal concentrations (Additional file 1: Table S4). For toxicity tests of the TBZ nanoformulations in soil, measured total soil concentrations for PCL-TBZ and NLC-TBZ at the beginning of the test (T0) mostly exceeded the aimed nominal concentrations, showing 130–226% and 126–187%, respectively, of the nominal concentrations (Additional file 1: Table S4). Soil concentrations only slightly decreased in the course of the experiment (96 h; average decrease by 13. ± 17.9%). In the microcosm experiment, TBZ concentrations were slightly higher at the beginning of the experiment for FOLICUR® and PCL-TBZ (148 and 163% of nominal concentration; Additional file 1: Table S5). However, in the course of the experiment (8 weeks), TBZ concentrations considerably decreased for FOLICUR® (44% decrease), whereas the concentrations stayed constant for PCL-TBZ (6% decrease).

Response of *C. elegans* to TBZ (a.s.) and formulations in water and soil

Toxicity of TBZ (a.s.) and TBZ formulations in water and soil

TBZ (a.s.) showed a dose-dependent inhibitory effect on the reproduction of *C. elegans* in water and soil (Fig. 1a), with considerably high toxicity in water (Table 1). Reproduction in control and solvent control was not significantly different, neither in water (72.8 ± 18.5 vs. 70.4 ± 9.5 offspring/test organism for control and solvent control, respectively) nor in soil tests (61.1 ± 10.5 vs. 63.1 ± 21.3 offspring/test organism for control and solvent control, respectively) ($p > 0.05$; one-way ANOVA). In water, effects did not exceed 63% inhibition at the highest concentration (measured C_{free}; 43 mg/L), which was already slightly higher than the reported water solubility of TBZ (36 mg/L). In soil, TBZ induced a maximum effect of 68% inhibition at a mean exposure concentration of 745 mg/kg dry soil.

All three TBZ formulations (FOLICUR®, PCL-TBZ, and NLC-TBZ) showed clear dose-dependent toxicity on the reproduction of *C. elegans* in water, with significantly stronger effects than could be expected from the measured freely dissolved concentrations of TBZ (Fig. 1b; *Z*-Test: $Z > 1.96$). While the no observed effect concentration (NOEC) values showed to be comparable or even higher than observed for TBZ tested as an a.s., EC_{50} values were considerably lower for the TBZ formulations (1.9, 2.5, and 4.5-fold for FOLICUR®, PCL-TBZ, and NLC-TBZ, respectively), if based on TBZ C_{free} (Table 1).

Table 1 EC_{50} and NOEC values of TBZ toxicity on *C. elegans*' reproduction applied as active substance (TBZ a.s.) in water and soil, and as commercial formulation (FOLICUR®) and nanoformulations (PCL-TBZ and NLC-TBZ) and the respective nanocarriers without TBZ in water; for the TBZ NF values are given in both units, freely dissolved TBZ concentrations and carrier nanoparticle concentrations;

Test item	TBZ (mg/L; mg/kg soil dw)		Particle concentration (particles/mL × 10^9)	
	EC_{50}	NOEC	EC_{50}	NOEC
TBZ (a.s.)				
Water	29.7 ± 4.1	<2.7	–	
Soil	711 ± ± 12.3	501	–	
FOLICUR®	16.0 ± 2.1[a]	5.2	–	
PCL-TBZ	11.5 ± 0.6[a]	5.6	5.7 ± 0.06[b]	4.4
NLC-TBZ	6.40 ± 0.4[a]	2.6	8.0 ± 0.29[b]	4.4
PCL-carrier	–		44.6 ± 4.0	8.6
NLC-carrier	–		11.7 ± 0.6	6.7

[a] Significantly different to TBZ (a.s.; water); [b]significantly different to carrier without TBZ (*Z*-Test; $Z > 1.96$)

Moreover, at C_{free} of TBZ (a.s.) < 30 mg/L (below the water solubility limit), maximal effects (94–100% inhibition of reproduction) of the three formulations could be observed (Fig. 1b), but not for the exposure in TBZ (a.s.; Fig. 1a).

In soil, *C. elegans* showed a similar response to FOLICUR® and PCL-TBZ as for TBZ (a.s.) if referring to the measured soil concentrations, with pronounced effects only occurring for TBZ soil concentrations exceeding the EC_{50} values derived from the soil tests with TBZ (a.s.; Fig. 1c). NLC-TBZ, however, showed considerably higher toxicity, with pronounced effects already at the lowest tested concentration (25% of EC_{50}; Fig. 1c).

Effects of NCs in water

Both types of NCs used for the TBZ NFs showed dose-dependent effects on the reproduction of *C. elegans* in the concentration range applied for NFs, while NLC nanocarriers exhibited considerably stronger effects than PCL nanocarriers (Fig. 1d). However, comparing the effects of NCs (without TBZ) with NCs in NFs (with TBZ) based on particle concentration, carriers in NFs still showed significantly stronger effects (Table 1; *Z*-Test: $Z > 1.96$).

Mixture toxicity modeling

For NLC-TBZ, observed and modeled dose–response curves perfectly matched, both showing an EC_{50} of 1.0 TUs, suggesting a concentration-additive mixture toxicity (Fig. 2). For PCL-TBZ, observed and modeled

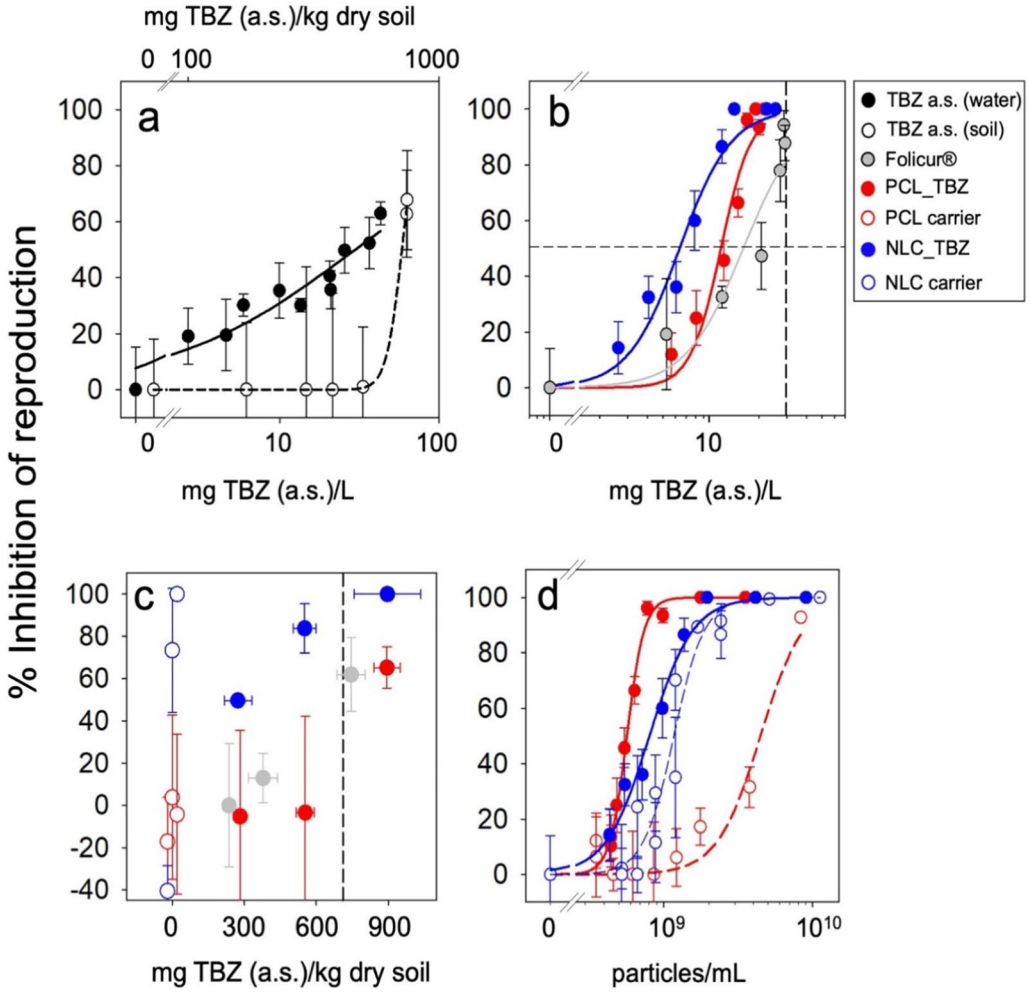

Fig. 1 Dose–response curves based on the measured TBZ concentrations for effects of TBZ (a.s.) on the reproduction of *C. elegans* after 96-h exposure to **a** TBZ (a.s.) in water and soil, **b** the commercial TBZ formulation FOLICUR® and the two nanoformulations PCL-TBZ and NLC-TBZ in water. Plot **c** shows the effects of the nanocarriers PCL and NLC with and without TBZ in soil based on the concentration of TBZ. The horizontal error bars stand for the standard deviation between the replicates of TBZ concentrations ($n = 3$). In Plot **c**, for each concentration of nanoformulations with TBZ, corresponding nanocarriers without TBZ were prepared using equivalent particle concentrations. Plot **d** shows the effects of the nanocarriers PCL and NLC with and without TBZ in water based on the number of particles; The curves were fitted with a logistic function (3 parameters) using SigmaPlot 12.0 (Systat Software Inc): **a** water: $r^2 = 0.90$, $p < 0.0001$; soil: $r^2 = 0.998$, $p < 0.0001$; **b** Folicur®: $r^2 = 0.91$, $p = 0.0009$; PCL-TBZ: $r^2 = 0.96$, $p < 0.0001$; NLC-TBZ: $r^2 = 0.97$, $p < 0.0001$; **c** PCL-TBZ: $r^2 = 0.99$, $p < 0.0001$; NLC-TBZ: $r^2 = 0.99$, $p < 0.0001$; PCL-carrier: $r^2 = 0.94$, $p < 0.0001$; NLC-carrier: $r^2 = 0.99$, $p < 0.0001$

dose–response curves considerably deviated from each other, suggesting that the observed toxicity cannot be explained by the combined effects of TBZ (a.s.) and NCs. While with the modeled dose–response curve, an EC_{50} of 0.94 TUs could be calculated, the dose–response curve for the observed effects gave an EC_{50} of 0.54 TUs (Fig. 2), suggesting a synergistic effect and rebut the assumption that the addition of TBZ and carrier effects can explain the observed effect of PCL- TBZ.

Microcosm study

At the beginning of the experiment, the Lufa soil showed a high nematode density (4472 ± 761 Ind/100 g dry soil; mean ± sd) and a high taxonomic diversity (22 ± 3 genera; Shannon–Wiener Index (log_2): 2.87 ± 0.35). The community was clearly dominated by plant parasitic nematodes (Hoplolaimidae: 49 ± 10%; Coslencus: 8 ± 4%; Paratylenchus: 6 ± 4%; Tylenchidae: 6 ± 2%), followed by bacterial feeders (several taxa: 13 ± 7%), omnivorous nematodes (several taxa: 7 ± 3%) and fungivorous nematodes (several

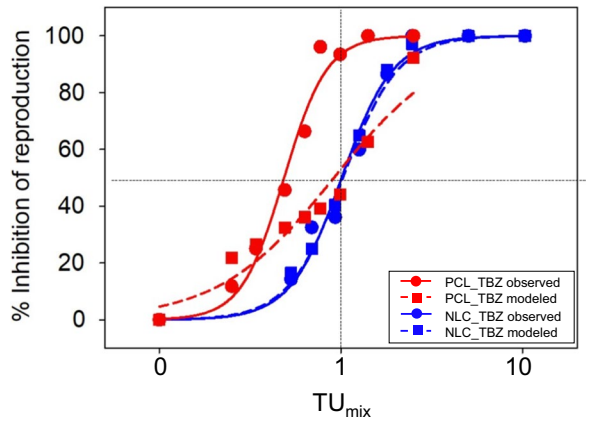

Fig. 2 Observed and modeled toxicity of TBZ NF (PCL-TBZ and NLC-TBZ): % inhibition of *C. elegans'* reproduction plotted against the sum of toxic units (TUmix = $TU_{carrier}$ + $TU_{TBZa.s.}$) calculated from carrier and TBZ concentrations and the respective EC_{50} values ($TU_{carrier\ or\ TBZ}$ = [carrier or TBZ]/ $EC_{50(carrier\ or\ TBZ)}$); mixture toxicity was modeled assuming independent action and effect addition

taxa: 7 ± 4%). With 2.72 (± 0.41) and 3.30 (± 0.27), the MI and MI25 were relatively high.

During the course of the study (8 weeks), the nematode community structure changed significantly in the control treatments (Fig. 3A; ANOSIM: $p < 0.05$). The microcosms treated with FOLICUR®, PCL-TBZ, and PCL nanocarriers also showed a significant change within the eight weeks of exposure, resulting in a significantly different community compared to the control treatment (Fig. 3A; ANOSIM: $p < 0.05$). Multivariate ANOSIM did not reveal a difference between the various treatments. However, the relative abundance of nematodes belonging to the family of Rhabditidae was considerably higher in FOLICUR® and PCL-TBZ treatments (Fig. 3D), with a significant difference to the control for PCL-TBZ ($p = 0.02$; one-way ANOVA, post hoc Dunnett). The difference in taxonomic composition between treatments and control resulted in a slight increase of total densities in PCL treatments (PCL-TBZ and PCL carriers; Fig. 3B), a slight decrease in the number of genera in all treatments (Fig. 3C), and a decrease of the MI in PCL-TBZ and FOLICUR®-treated microcosms (Fig. 3E) and an increase of MI25 in the PCL-TBZ treated microcosms (Fig. 3E). However, due to the high variability of the number of individuals, number of genera, and MI, these trends were not statistically significant ($p > 0.05$).

Discussion

Characteristics of nanoformulations

The physical characterizations of prepared nanoparticles (in stock suspensions) confirmed that nanoparticles within the "nano-range" of up to several hundred nanometers are consistent with nanopesticides definitions [9, 25,

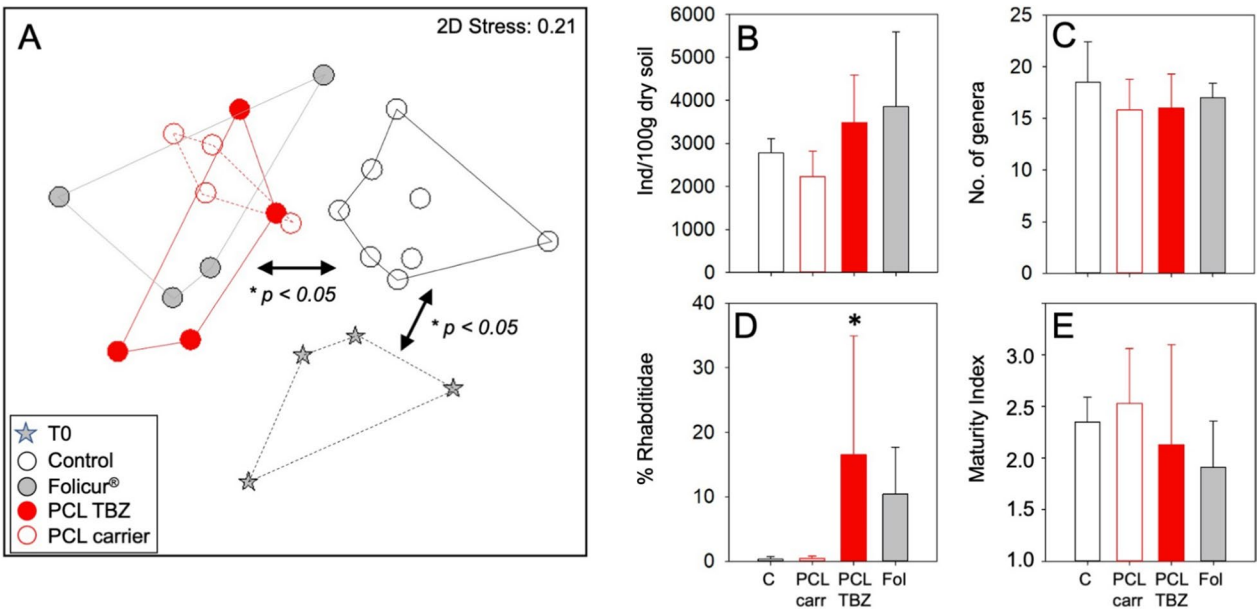

Fig. 3 Nematode community structure in soil microcosms treated with water (control; C), FOLICUR® (Fol), TBZ NF (PCL TBZ) and PCL nanocarriers (PCL carr) after 8 weeks of exposure: **A** nMDS-plot based on square-root transformed relative nematode genus abundances; symbols represent single replicate microcosms ($n = 4$; controls: $n = 8$); arrows mark significant changes of genus composition within the course of the experiment (T0 vs Control) and differences between the control and all treated microcosms; **B** total nematode densities (individuals/100 g dry soil; mean ± sd; $n = 4$); **C** number of genera (mean ± sd; $n = 4$); **D** % Rhabditidae (mean ± sd; $n = 4$; * = significantly different to control); **E** Maturity Index (MI; mean ± sd; $n = 4$)

65], aligning with previous research on nanopesticides with PCL (17) and NLC [57]. Other key physical properties such as PI and ζ potential were evaluated, indicating high uniformity and stability of the nanoparticles, with PI values around 0.2 and ζ potential around (±) 30 mV, signaling optimal dispersion and stability [13, 57]. Additionally, the EE of the a.s. in nanoparticles ranged from 91 to 98%, highlighting the effective association of TBZ with the NCs. These findings underscore that the NCs' hydrophobic composition remarkably favors the binding of TBZ that ended in the perfect association across all NFs [13, 20].

In fresh Lufa 2.2 soil, which was used for the microcosm study, the unchanged TBZ concentration within the eight-week study, if applied as nanoformulation (PCL-TBZ), shows that the nanoformulation can protect the a.s. from degradation. The lower degradation of a.s. encapsulated in NCs has been demonstrated in different studies [31, 66]. Degradation is demonstrated as the main dissipation for TBZ [31].

Toxicity of TBZ (a.s. and commercial formulation)

In the present study, TBZ was tested in relatively high concentration to simulate the worst-case scenario in cumulative release circumstances at the high application of TBZ [48, 50]. In the most extreme FOCUS scenario (Forum for the co-ordination of pesticide fate models and their use. D2: ditch, spray application to cereals), the estimated concentration of TBZ in surface water (predicted environmental concentration in surface water) was found to be 2.47 µg/L [67, 68]. Moreover, according to the literature, TBZ levels in surface water have been reported to reach between 175 and 200 µg/L [69]. Furthermore, evidence from a study conducted between 2009 and 2012 revealed that TBZ concentrations at a vineyard outlet in the Layon catchment in France peaked at 357 µg/L [70]. The soil DT_{50} reported for TBZ is 25–365 days [49]. Considering the high persistence and high to medium mobility of TBZ in water and soil [67], the importance of this study is highlighted. In the present study, TBZ elicited 96 h- EC_{50} value of 29.7 mg a.s./L in water and 711 mg a.s./kg in soil (Table 1). Due to the limited water solubility of TBZ (36 mg/L, 25 ˚C), freely dissolved TBZ concentrations did not exceed 47 mg/L even at a nominal concentration of 200 mg/L, in spite of the presence of solvent (0.5% acetone; see Additional file 1: Table S3). Therefore, in both water and soil, the maximum effect could not reach 100% (< 70% inhibition of reproduction). Moreover, the NOEC value in water was < 2.7 mg/mL and 501 mg/kg in soil. In a study on *C. elegans* on agar plate, a NOEC value of 1000 µg/L and LOEC value of 2500 µg/L were determined for *C. elegans* [68], indicating the high toxicity of TBZ.Compared to *C. elegans*, aquatic organisms like *Daphnia magna* and *Danio rerio* have shown higher sensitivity to TBZ. For example, *D. magna* exhibited a 48-h EC_{50} between 2.37 and 6.2 mg/L and a more sensitive 21-day reproduction EC_{50} at 0.7 mg/L [50, 71, 72]. *Danio rerio*'s 96-h LC_{50} values range from 0.89 to 9.7 mg/L, indicating significant toxicity.

The EFSA report [67] on TBZ shows that for *Eisenia fetida*, the 8-week NOEC and 14-day LC_{50} values are 10 mg/kg and 1381 mg/kg dry soil, respectively. Chen et al. [73] reported LC_{50} values for *E. fetida* at 746.3 mg/kg and 287 mg/kg for 7 and 14 days. Comparatively, this study's 4-day EC_{50} for *C. elegans* is 711 mg/kg dry soil, aligning closely with values for *E. fetida*.

TBZ targets a critical site involved in cell membrane construction across bacteria, fungi, plants, and animals, indicating its potential to disrupt both biological functions, such as feeding and assimilation, and broader ecological processes [47, 69]. For example, TBZ significantly affects feeding and energy storage in *Daphnia magna* at concentrations over 410 µg/L after 24 h of exposure [74], highlighting its metabolic impact. Research on *C. elegans* has shown that exposure to TBZ, even at concentrations as low as 0.01 µg/L, can cause reproductive and developmental defects, including reduced brood size [47]. This disruption is speculated to be due to TBZ's interference with crucial enzymes such as cytochrome P450 (CYP)17, essential for steroid hormone synthesis in vertebrates, leading to decreased progesterone and estrogen levels [47]. Similar toxic effects, through interaction with the endocrine system and implications for development in both invertebrates and mammals, have been suggested [68]. Additionally, TBZ's inhibition of CYP19a expression in zebrafish disrupts steroid hormone biosynthesis, reducing fertility by lowering 17β-estradiol levels [53]. These findings highlight TBZ's wide-ranging effects on metabolism and development across different species and could potentially apply to *C. elegans* in the present study. The large difference between TBZ toxicity to *C. elegans* in water and soil (30 mg/L vs. 711 mg/kg) might be due to the binding of TBZ to organic soil particles, lowering the bioavailability of the compound [31]. Taking a K_{OC} of 1536 (EPISuite) and a f_{OC} of 0.0171 of the Lufa soil (% TOC: 1.71) the EC_{50} of 711 mg/kg dw refers to an estimated porewater EC_{50} of 28.2 mg/L [75]. The good agreement of the porewater-based EC_{50} derived via soil exposure with the EC_{50} derived from water exposure (29.7 mg/L) confirms the assumption that the toxicity of TBZ in soil was mainly caused by freely dissolved TBZ. Moreover, Haegerbaeumer et al. [44] showed that the toxicity of the fungicide fludioxonil to *C. elegans* was comparable for soil and water exposure when referring to porewater concentrations, suggesting the porewater as the main uptake route. This finding also agrees with

studies on the toxicity of fludioxonil on nematodes in freshwater sediments [76].

Recognizing the potential for formulations to manifest higher toxicities compared to their respective a.s., it is imperative to conduct toxicity assessments on the pesticide formulations for a comprehensive evaluation [77]. Moreover, the use of the commercial formulation is relevant because plant protection products are deployed in the environment as formulations, potentially introducing both the a.s. and their additives [69]. In Fojtová et al. study [31], commercial formulation typically delayed degradation of a.s. (with DT_{50} extending by 1.5 to 2 times relative to a.s.) and resulted in increased soil residue levels in comparison to the active substance. Formulations contain additives (used to improve their functionality, e.g., solubility) that may induce excess toxicity in the organism [78]. In the present study, FOLICUR® inhibited reproduction more than TBZ (a.s.) in water with 96 h-EC_{50} = 16 mg/L. FOLICUR® is a suspoemulsion, with oil being a major component of the formulation (20%) as inert ingredient [79]. Although we were not able to test the effect of this unknown component on the reproduction of *C. elegans,* the effects of a high density of lipid droplets, analogous to the effects of the NCs, can be considered as a possible cause for the observed excess toxicity. It cannot be excluded that FOLICUR® also comprises additional biocidal components, namely 1,2-benzisothiazol-3(2H)-one (BIT) and a combination of 5-chloro-2-methyl-3(2H)-isothiazolone (< 0.0015%) and 2-methyl-2H-isothiazol-3-one, (< 0.05%). According to the literature, BIT` LC_{50} values for *D. magna* and zebrafish in freshwater environments are 1.03 and 1.5 mg/L, respectively, and it could significantly reduce the growth of algae like *Scenedesmus* sp. LX1, *Chlorella* sp with 72-h EC_{50} values of 1.70, 0.41 and 1.16 mg/L, respectively [80]. BIT's behavior in soil shows it degraded quickly (t½ = 7.2 h) in terrestrial environments, with any resulting metabolites being short-lived and posing considerably less toxicological risk [81]. However, further research is essential to gain an understanding of the impact of these additives on nematodes. Also, a slightly higher toxicity of Folicur EW 250 compared to TBZ (a.s.) was reported for *E. fetida* without any provided speculation regarding the cause of this toxicity [67].

Toxicity of TBZ NFs and their corresponding NCs

The nanoformulations, PCL-TBZ and NLC-TBZ, displayed distinct toxicity patterns in both water and soil on reproduction. Compared to TBZ (a.s.), both NFs were considerably more toxic in water (factor 2.5 and 4.5 for PCL-TBZ and NLC-TBZ, respectively; Fig. 1b; Table 1). In soil, PCL-TBZ showed no toxicity until the highest concentration, comparable to the toxicity of TBZ (a.s.) (Additional file 1: Table S3). In contrast, NLC-TBZ showed considerably higher toxicity than TBZ (a.s.) and PCL-TBZ. However, a considerable part of the toxic effect of the TBZ NFs might have been contributed by the intrinsic toxicity of the NCs themselves, while *C. elegans* responded more sensitively to NLC than to PCL nanocarriers, both in water and soil (Table 1, Fig. 2). Different effect mechanisms might explain the nanocarrier effect. For both types of NCs, a physical particle effect can be assumed that has already been observed for *C. elegans* after exposure to high concentrations of polystyrene particles. Mueller et al. [82] suggested food depletion as a major effect mechanism, as the dilution of the food (bacteria) with particles of similar size led to a significantly lower uptake of nutritious bacteria [83]. This is supported by Lu et al. [47]. The authors explained that the adverse effect seen on *C. elegans* could be linked to a diminished feeding capability necessary for securing enough nutrients for growth, reproduction, and sustaining metabolism. Additionally, Mueller et al. [82] reported EC_{50} values for polystyrene beads at 8 and 140×10^9 beads/mL, depending on the size of the beads, 0.5 to 0.1 µm diameter, respectively. This agrees well with the observed effects of the NLC and PCL nanocarriers in the present study (EC_{50}: 12 and 45×10^9 particles/mL, respectively; Table 1) with a size of 0.32 and 0.26 µm, respectively (Additional file 1: Table S2).

However, as NLC showed considerably stronger effects on the nematodes than PCL, in both water and soil tests, other effect mechanisms, such as chemical-induced toxicity, could also be considered. For lipid nanoparticles, toxicity can be influenced by the presence of toxic elements and radical species generation (reactive oxygen species and other free radicals) that contribute to cytotoxicity [84]. Glyceryl monostearate was found to be responsible for the cytotoxicity of solid lipid nanoparticles (SLN) and NLC [84]. Moreover, the lipid compositions, as well as surfactants, emulsifiers, and stabilizers used in NLC preparation, might be important factors for the toxicity of lipid nanoparticles [84]. In the current study, the lipid phase was made up of solid lipid (glycerol tripalmitate) and liquid lipid (Myritol 318), where the ecotoxicity data for these two lipids are scarce considering the general belief that the lipids are biocompatible [84]. In a study by Albuquerque et al. [85] different concentrations of SLN (with similar compositions as our tested NLC) with and without atrazine were tested on *Chironomus sancticaroli* larvae, where atrazine NFs and their corresponding NCs (without atrazine) caused mortality and biochemical alterations, indicating potential toxicity, while the effects of atrazine as a.s. showed no lethal effects.

Similarly, the toxicity study of SLN on *C. elegans* demonstrated comparable mortality outcomes between the SLN formulation encapsulating atrazine and their corresponding NCs. In contrast, *C. elegans* exposed to the herbicide (a.s.) exhibited no mortality, pointing to the nanoparticles' composition as a key toxicity factor possibly due to unique mechanisms of interaction or internalization with the organisms [32]. Furthermore, research by Gomez et al. [86] on the effects of nano-atrazine on gene transcripts involved in secretion, translocation, and vesicle trafficking shed light on a potential nano-specific pathway for uptake and cellular transport, indicating that the nanoparticles may facilitate a distinct mode of entry and distribution within cells. In the present study, in soil, NCs (without TBZ) likely contributed to the higher toxicity of NFs compared to TBZ (a.s.). We only observed excess toxicity for NLC-TBZ, where the carrier itself showed considerable inhibitory effects on the nematodes' reproduction (Fig. 1c). The slow degradation of lipid nanoparticles in soil, as documented by Ayoubi et al. [87], may further contribute to the observed toxicity in *C. elegans*. In a related context, the toxicity assessment of NLC-TBZ (at concentrations below the TBZ solubility limit) revealed high toxicity on the immobilization of *D. magna* in the AdaM medium. Importantly, this toxicity was attributed to the release of TBZ into the test medium rather than the corresponding NCs [72]. Contrary to these results and our results of NLC, SLN incorporating a mixture of atrazine and simazine demonstrated a reduction in cytotoxicity to 3T3 rat fibroblast cells and phytotoxicity to corn plants (*Zea mays*) [57]. These results emphasize that the toxicity of nanoparticles, particularly lipid nanoparticles, is a complex and multifaceted issue that demands careful evaluation and consideration of various factors. The observed low toxicity of PCL-TBZ on *C. elegans* in soil may be due to slower TBZ release from PCL NCs [31, 88] and a slower degradation rate of PCL within the exposure period [16, 17, 31]. Studies have shown that PCL degrades more slowly in soil than in other environments, such as compost, with its biodegradation rate ranging from a few months to several years. This variability is largely dependent on the molecular weight of PCL and specific environmental conditions that influence its degradation [16, 17, 89].

Combined toxicity of TBZ (a.s.) and NCs

To test if the observed effects of both NFs are a result of combined toxicity of nanocarriers and TBZ (a.s.), mixture toxicity models using effect addition (assuming independent action) were applied to the data. For NLC-TBZ, the mixture model perfectly predicted the observed effect (Fig. 2) so that the combined toxicity of NCs and TBZ (a.s.) could be confirmed. In contrast, the PCL-TBZ formulation exhibited a synergistic effect, where the observed toxicity was significantly higher than what would be expected from the combined effects of the nanocarriers and TBZ a.s. (with an EC_{50} value of 0.54 TUs). This suggests that the interaction between PCL NCs and TBZ results in enhanced toxicity beyond simple additive effects. This increased toxicity could be due to not only the bioavailability of freely dissolved TBZ, but also TBZ encapsulated within the PCL NCs, which might be released inside the nematodes' gut upon ingestion, indicating a more complex interaction mechanism at play. This agrees with other studies mentioning an increased bioavailability of a.s. by NCs and increasing the uptake of a.s. and organism gut digestion [31, 90].

For the commercial TBZ formulation, FOLICUR®, combined toxicity of TBZ (a.s.) and the oil used for creating the emulsion could not be modeled, as it was not possible to test the toxicity of the exact oil emulsion on *C. elegans.* However, the slightly higher toxicity of FOLICUR® on the nematodes' reproduction compared to the TBZ (a.s.) might be partly induced by the emulsifier. The available literature suggests that the toxicity of FOLICUR® on the nematodes' reproduction compared to the TBZ (a.s.) might be partly induced by the emulsifier. Still, the exact mechanism of action of the emulsifier is not specified [72, 91]. Further research may be needed to determine the mechanism of action of the emulsifier in FOLICUR® (explained broader in section "Toxicity of TBZ (a.s. and commercial formulation)").

These results highlight the complexity of assessing the toxicity of NFs and the importance of considering not only the a.s. but also the NCs and other components present in the formulations [25]. It demonstrates that interactions within NFs can significantly alter the toxicity profile, emphasizing the need for detailed toxicological evaluations that account for potential synergistic effects.

Long-term effects of TBZ NF in microcosms

Although measured TBZ soil concentrations in the microcosm test (405 and 551 mg/kg dw for FOLICUR® and PCL-TBZ) were below or comparable to the NOEC derived for *C. elegans* in soil, we expected that the native soil nematode fauna in Lufa soil respond to TBZ with a significant change in the taxonomic composition, due to a considerably longer exposure time (8 weeks vs. four days) and a multispecies scenario with a larger range of sensitivity towards TBZ. In a similar setting, nematode communities were shown to respond more sensitively to fungicides in microcosms than *C. elegans* in a single-species test [44, 45]. In the present study, the nematodes responded with a significant change in the taxonomic composition compared to the untreated control; however, this happened in all treatments, even if only NCs

were present without TBZ (Fig. 3a). Thus, the major effects seem to have been caused by the NCs or emulsifier (oil in FOLICUR®), rather than by TBZ (a.s.). However, the effects of TBZ in the PCL-TBZ and FOLICUR® treatments cannot be excluded, as certain changes (e.g., the relative increase of Rhabditidae) only occurred in treatments containing TBZ, which also led to a slight decrease of the MI, a stress index for nematode communities. Thus, also under more realistic settings in the microcosms (multispecies; long-term exposure), a combined effect of TBZ and NCs might have been responsible for the observed effects on the native soil nematodes.

Gradual increase of bioaccumulation of TBZ encapsulated in PCL NCs within a microcosm study on earthworms in Lufa 2.4 (comparable TOC = 1.9% to the present study' soil type TOC = 1.71%) was reported which was attributed to nanoformulated pesticides being more readily available to earthworms reflecting the gradual release of the a.s. from NCs into the soil's pore water [31, 66]. Similarly, Firdaus et al. [92] reported a 50% higher bioaccumulation of nanoformulated bifenthrin in earthworms compared to its a.s. and commercial formulations, indicating that NFs not only increase bioaccumulation but also modify the uptake and elimination dynamics in earthworms. Notably, nano-encapsulated bifenthrin primarily remained in the earthworms' gut, unlike the conventional formulation, which was absorbed into their tissue. This suggests a change in the exposure mechanism, likely due to the a.s. being encapsulated within NCs that act as a protective barrier. And changes depend on the specific compound, soil characteristics, and duration of exposure [88]. Hence, the slow release of TBZ into the soil pore water, coupled with the bioavailability of nanopesticides as a complex (pesticide within NCs), could explain the observed alterations in the native soil nematode fauna in this study.

Different components used in the preparation of PCL, in longer time exposure may negatively affect the nematode community. Myritol 318 is a blend of triglycerides derived from caprylic and capric acids. It is considered a non-toxic fatty acid, but to the best of our knowledge, there is no specific information available on the toxicity of Myritol 318 to nematodes. Free fatty acids like caprylic and capric acids were shown to significantly reduce *Meloidogyne incognita* (root-knot nematodes) reproduction and juvenile mortality [93]. In an investigation into the toxicity of nonionic surfactants used in polycaprolactone (PCL) compositions, specifically Tween 80, it was found that at a concentration of 0.05% (equivalent to 500 mg/L), there were no adverse effects observed on the reproduction of *C. elegans*. (unpublished data).

These microcosm results suggest that the toxicity and exposure dynamics of the treatments, potentially influenced by the differential release rates of a.s. in different environmental matrices, could impact nematode communities. Understanding the internal exposure of nematodes to nanoparticles, as highlighted by the variable release rates of a.s. in different conditions (e.g., cellular compartments versus soil pore water: quicker a.s. release in cells and slower in soil pore water due to variations in pH levels and cellular enzyme activity), could provide insights into the relationship between nanoparticles' properties and observed toxic effects [25] and may explain the significant nematode community shifts observed in the present study. This complexity underlines the need for a deeper investigation into how PCL nanoparticles interact with nematodes.

Implications for the risk assessment of TBZ NFs

Establishing a body of knowledge related to the combined effects of nanopesticides will be helpful for regulatory agencies in the environmental risk assessment of these compounds. Therefore, it is important to consider the risks of a combined (and potentially synergistic) toxicity of a.s. and formulation on non-target species to indicate response patterns at different levels..

In assessing mixture (combined) toxicity, the common approach involves using the concentration addition (CA) model for chemicals with similar toxicity mechanisms and the independent action (IA) model for chemicals with entirely different mechanisms. However, for environmentally realistic mixtures containing substances with varying modes of action, the application of CA and IA estimates has garnered significant attention in the scientific literature [71]. The mixture analysis for NLC-TBZ revealed the concentration addition model with EC_{50} TU mix = 1.0.

The results of the water test with the tested compounds indicate that the NOEC of TBZ toxicity assessment is lower than the NOEC for other formulations. This suggests that TBZ may have a greater potential to impact *C. elegans* in lower concentrations compared to the other tested formulations. Similar to the current results, reported NOEC for TBZ on toxicity tests with *D. magna* and *C. elegans* were also low (< 0.4 and 1 mg/L, respectively), showing the high toxicity of TBZ [67, 68, 71]. In the present study, the lower NOEC for TBZ (< 2 mg/L) was followed closely by the recorded NOEC for NLC-TBZ. The NOEC is an important parameter in ecological risk assessments, as it represents the highest tested concentration of a substance at which no adverse effects are observed on a specific organism or ecological community.

Conclusions

This study revealed combined toxicity in the tested nanopesticides arising from the combination of TBZ as the a.s. and the NCs employed in its NFs, which led to an increased toxic effect of these NFs compared to TBZ alone. For TBZ encapsulated in NCs with high toxicity potential, such as NLC, the effects of the NF can be explained by effect addition. However, for NCs like PCL, which exhibit lower toxicity, synergistic effects are observed on *C. elegans*. Our study highlights that risk assessments based solely on the a.s. (TBZ) are inadequate for its NFs, as evidenced in our studies across aqueous and soil matrixes and under both short- and long-term exposures. This underscores the necessity, as also emphasized by other researchers, for a comprehensive and robust risk assessment framework specifically for nanopesticides [25, 26, 94].

Overall, this study offers important perspectives on the ecological impacts of various TBZ formulations, with an emphasis on NFs. This information is crucial as it adds to the growing body of evidence necessary to evaluate the ecological safety of nanopesticides, which are increasingly being considered as alternatives to their traditional counterparts due to their potential for lower dosages and targeted delivery. The outcomes presented here are based on laboratory-scale exposure scenario, which may differ under varying conditions. In addition, the toxicity of the formulations was assessed at high concentrations without accounting for the possibility that nanopesticides could be more effective at lower dosages. Therefore, further studies on the bioactivity and toxicity of nanopesticides are essential for their safe and sustainable integration into agricultural practices. This should be supported by harmonized testing protocols and stringent regulations to assess their risks accurately.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-024-00879-9.

Additional file 1. S1. Synthesis of nanopesticides. **S2**. Physical characterization of nanoformulations and nanocarriers. **S3**. Morphology of nanoparticles. **S4**. Chemical characterization of nanoformulations. **S5**. Nematode growth medium. **S6**. Preparation of food suspension for nematode. **Table S1**. Overview of experiments with respective test conditions. **Table S2**. Characterization of stock nanoformulations. **Tables S3**, **S4** and **S5**. Measured TBZ concentrations in water, soil, and microcosm tests, respectively. **Figure S1**. Transmission electron microscopy micrographs of nanoparticles. **Figures S2** and **S3**. Size distributions of nanoparticles in poly-ε-caprolactone and nanostructured lipid nanocarriers, respectively.

Acknowledgements

This research was funded by GAČR project GA18-19324S. Authors thank the RECETOX Research Infrastructure (No LM2023069) financed by the Ministry of Education, Youth and Sports, and the European Union's Horizon 2020 research and innovation programme under grant agreement No 857560 for supportive background. This publication reflects only the authors view, and the European Commission is not responsible for any use that may be made of the information it contains. R. G. would like to thank the São Paulo Research Foundation (Grant #2022/03219-2), the National Council for Scientific and Technological Development (Grant #310846/2022-6), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. We are grateful for the helpful discussions on mixture toxicity with Prof. Dr. Lennart Weltje.

Author contributions

ME: Conceptualization, methodology, formal analysis, data curation, writing—original draft, writing—review and editing, funding acquisition. JH: Conceptualization, resources, writing—review and editing, supervision, funding acquisition. JK: Resources, formal analysis, writing—review and editing. RG: Writing—review and editing. ZHB: Formal analysis, writing—review and editing. NR: Formal analysis, writing—review and editing. SH: Conceptualization, methodology, formal analysis, resources, writing—review and editing, supervision.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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Received: 28 December 2023 Accepted: 28 February 2024
Published online: 07 March 2024

References

1. Silva V, Mol HGJ, Zomer P, Tienstra M, Ritsema CJ, Geissen V (2019) Pesticide residues European agricultural soils – a hidden reality unfolded. Sci Total Environ 653:1532–1545
2. Sharma A, Kumar V, Shahzad B, Tanveer M, Sidhu GPS, Handa N et al (2019) Worldwide pesticide usage and its impacts on ecosystem. S N Appl Sci 1:1446
3. Tudi M, Ruan HD, Wang L, Lyu J, Sadler R, Connell D et al (2021) Agriculture development, pesticide application and its impact on the environment. Int J Environ Res Public Health 18(3):1–24
4. Lowry GV, Avellan A, Gilbertson LM (2019) Opportunities and challenges for nanotechnology in the agri-tech revolution. Nat Nanotechnol 14(6):517–522
5. Sabzevari S, Hofman J (2022) A worldwide review of currently used pesticides' monitoring in agricultural soils. Sci Total Environ 812:152344
6. European Parliament (2023) Pesticides: MEPs want a drastic cut in use of chemical pesticides. Press release. 20231023IPR08130. Press switchboard number (32–2) 28 33000.
7. Kah M, Kookana RS, Gogos A, Bucheli TD (2018) A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. Nat Nanotechnol 13(8):677–684
8. Wang D, Saleh NB, Byro A, Zepp R, Sahle-Demessie E, Luxton TP et al (2022) Nano-enabled pesticides for sustainable agriculture and global food security. Nat Nanotechnol 17(4):347–360
9. Nuruzzaman M, Rahman MM, Liu Y, Naidu R (2016) Nanoencapsulation, nano-guard for pesticides: a new window for safe application. J Agric Food Chem 64(7):1447–1483
10. Kah M, Hofmann T (2014) Nanopesticide research: current trends and future priorities. Environ Int 63:224–235

11. Grillo R, Chirakkuzhyil Abhilash P, Fernandes Fraceto L (2016) Nanotechnology applied to bio-encapsulation of pesticides. J Nanosci Nanotechnol 16(1):1231–1234
12. An C, Sun C, Li N, Huang B, Jiang J, Shen Y et al (2022) Nanomaterials and nanotechnology for the delivery of agrochemicals: strategies towards sustainable agriculture. J Nanobiotechnol 20(1):1–19
13. Grillo R, dos Santos NZP, Maruyama CR, Rosa AH, De Lima R, Fraceto LF (2012) Poly(ε-caprolactone) nanocapsules as carrier systems for herbicides: physico-chemical characterization and genotoxicity evaluation. J Hazard Mater 231–232:1–9
14. Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A (2004) Poly-ε-caprolactone microspheres and nanospheres: an overview. Int J Pharm 278(1):1–23
15. Dash TK, Konkimalla VB (2012) Poly-ε-caprolactone based formulations for drug delivery and tissue engineering: a review. J Control Release 158(1):15–33
16. Heimowska A, Morawska M, Bocho-Janiszewska A (2017) Biodegradation of poly(**ϵ**-caprolactone) in natural water environments. Pol J Chem Technol 19:120–126
17. Bartnikowski M, Dargaville TR, Ivanovski S, Hutmacher DW (2019) Degradation mechanisms of polycaprolactone in the context of chemistry, geometry and environment. Prog Polym Sci 96:1–20
18. Pereira AES, Grillo R, Mello NFS, Rosa AH, Fraceto LF (2014) Application of poly(epsilon-caprolactone) nanoparticles containing atrazine herbicide as an alternative technique to control weeds and reduce damage to the environment. J Hazard Mater 268:207–215
19. Campos EVR, Oliveira JL, Da Silva CMG, Pascol M, Pasquoto T, Lima R et al (2015) Polymeric and solid lipid nanoparticles for sustained release of carbendazim and tebuconazole in agricultural applications. Sci Rep 5:1–14
20. López-Cabeza R, Kah M, Grillo R, Koutný M, Salač J, Bílková Z et al (2022) Tebuconazole and terbuthylazine encapsulated in nanocarriers: preparation, characterization and release kinetics. Environ Sci: Nano 9:1427–1438
21. Müller RH, Radtke M, Wissing SA (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv Drug Deliv Rev 54:131–155
22. Jaiswal P, Gidwani B, Vyas A (2016) Nanostructured lipid carriers and their current application in targeted drug delivery. Artif Cells Nanomed Biotechnol 44(1):27–40
23. Oliveira HC, Stolf-Moreira R, Martinez CBR, Grillo R, De Jesus MB, Fraceto LF (2015) Nanoencapsulation enhances the post-emergence herbicidal activity of atrazine against mustard plants. PLoS ONE 10(7):1–12
24. Pontes MS, Antunes DR, Oliveira IP, Forini MML, Santos JS, Arruda GJ et al (2021) Chitosan/tripolyphosphate nanoformulation carrying paraquat: insights on its enhanced herbicidal activity. Environ Sci: Nano 8(5):1336–1351
25. Kookana RS, Boxall ABA, Reeves PT, Ashauer R, Beulke S, Chaudhry Q et al (2014) Nanopesticides: guiding principles for regulatory evaluation of environmental risks. J Agric Food Chem 62(19):4227–4240
26. Li L, Xu Z, Kah M, Lin D, Filser J (2019a) Nanopesticides: a comprehensive assessment of environmental risk is needed before widespread agricultural application. Environ Sci Technol 53:7923–7924
27. Grillo R, Fraceto LF, Amorim MJB, Scott-Fordsman JJ, Schoonjans R, Chaudhry Q (2021) Ecotoxicological and regulatory aspects of environmental sustainability of nanopesticides. J Hazard Mater 404:124148
28. Lowry GV, Gregory KB, Apte SC, Lead JR (2012) Transformations of nanomaterials in the environment. Environ Sci Technol. https://doi.org/10.1021/es300839e
29. Lowry GV, Espinasse BP, Appala R, Richardson C, Reinsch BC, Bryant LD et al (2012) Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland. Environ Sci Technol 46(13):7027–7036
30. Tiede K, Hassellöv M, Breitbarth E, Chaudhry Q, Boxall ABA (2009) Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J Chromatogr A 1216(3):503–50931
31. Fojtová D, Vašíčková J, Grillo R, Bílková Z, Šimek Z, Neuwirthová N et al (2019) Nanoformulations can significantly affect pesticide degradation and uptake by earthworms and plants. Environ Chem 16(6):470–481
32. Jacques MT, Oliveira JL, Campos E, Fraceto LL, Silva Ávila D (2017) Safety assessment of nanopesticides using the roundworm *Caenorhabditis elegans*. Ecotoxicol Environ Saf 139:245–253
33. Bongers T, Ferris H (1999) Nematode community structure as a biomonitor in environmental monitoring. Trends Ecol Evol 14:224–228
34. Van den Hoogen J, Geisen S, Routh D, Ferris H, Traunspurger W, Wardle DA et al (2019) Soil nematode abundance and functional group composition at a global scale. Nature 572:194–198
35. Höss S, Gansfort B, Traunspurger W (2022) Nematodes as ecological and environmental indicators. In: Glazer I, Shapiro-Ilan D, Sternberg PW (eds) Nematodes as Model Org. CABI Publishing, Wallingford, p 376
36. Félix MA, Braendle C (2010) The natural history of *Caenorhabditis elegans*. Curr Biol 20(22):965–969
37. ASTM (2001) Standard guide for conducting laboratory soil toxicity tests with the nematode *Caenorhabditis elegans*; ASTM E2172–01. American Society for Testing and Materials, West Conshohocken
38. ISO (2020) Water and soil quality—Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda) (2nd edition); ISO 10872:2020; International Organization for Standardization: Geneva, Switzerland.
39. Wittkowski P, Marx-Stoelting P, Violet N, Fetz V, Schwarz F, Oelgeschläger M et al (2019) *Caenorhabditis elegans* as a promising alternative model for environmental chemical mixture effect assessment a comparative study. Environ Sci Technol 53(21):12725–12733
40. Anderson GL, Boyd WA, Williams PL (2001) Assessment of sublethal endpoints for toxicity testing with the nematode *Caenorhabditis elegans*. Environ Toxicol Chem 20:833–838
41. Ristau K, Akgül Y, Bartel AS, Fremming J, Müller M-T, Reiher L et al (2015) Toxicity in relation to mode of action for the nematode *Caenorhabditis elegans*: acute to chronic ratios and quantitative structure-activity relationships. Environ Toxicol Chem 34:2347–2353
42. Höss S, Reiff N, Nguyen HT, Jehle JA, Hermes H, Traunspurger W (2014) Small-scale microcosms to detect chemical induced changes in soil nematode communities – effects of crystal proteins and Bt-maize plant material. Sci Total Environ 472:662–671
43. Höss S, Reiff N, Traunspurger W, Helder JA (2021) On the balance between practical relevance and standardization – testing the effects of zinc and pyrene on native nematode communities in soil microcosms. Sci Total Environ 788:147742
44. Haegerbaeumer A, Raschke R, Reiff N, Traunspurger W, Höss S (2019) Comparing the effects of fludioxonil on non-target soil invertebrates using ecotoxicological methods from single-species bioassays to model ecosystems. Ecotoxicol Environ Saf 183:109596
45. Höss S, Reiff N, Asekunowo J, Helder J (2022) Nematode community of a natural grassland responds sensitively to the broad-spectrum fungicide mancozeb in soil microcosms. Environ Toxicol Chem 41:2420–2430
46. Muñoz-Leoz B, Ruiz-Romera E, Antigüedad I, Garbisu C (2011) Tebuconazole application decreases soil microbial biomass and activity. Soil Biol Biochem 43(10):2176–2183
47. Lu Q, Bu Y, Ma L, Liu R (2020) Transgenerational reproductive and developmental toxicity of tebuconazole in *Caenorhabditis elegans*. J Appl Toxicol 40(5):578–591
48. European Commission (2007) The use of plant protection products in the European Union. Data 1992–2003. ISBN 92-79-03890-7. http://ec.europa.eu/eurostat/documents/3217494/5611788/KS-76-06-669-EN.PDF/36c156f1-9fa9-4243-9bd3-f4c7c3c8286a?version=1.0.
49. Lewis KA, Tzilivakis J, Warner D, Green A (2016) An international database for pesticide risk assessments and management. Hum Ecol Risk Assess Int J 22(4):1050–1064
50. Tofan L, Niță V, Nenciu M, Coatu V, Lazăr L, Damir N et al (2023) Multiple assays on non-target organisms to determine the risk of acute environmental toxicity in tebuconazole-based fungicides widely used in the Black Sea Coastal Area. Toxics 11(7):597
51. Chen X, Zhu Q, Li X, Huang T, Wang S, Wang Y, Chen X, Lin Zh, Ge R (2019) Pubertal exposure to tebuconazole increases testosterone production via inhibiting testicular aromatase activity in rats. Chemosphere 230:519–526
52. Taxvig C, Hass U, Axelstad M, Dalgaard M, Boberg J, Raun Andeasen H, Vinggaard A (2007) Endocrine-disrupting activities in vivo of the fungicides tebuconazole and epoxiconazole. Toxicol Sci 100(2):464–473
53. Li S, Sun Q, Wu Q, Gui W, Zhu G, Schlenk D (2019b) Endocrine disrupting effects of tebuconazole on different life stages of zebrafish (*Danio rerio*). Environ Pollut 249:1049–1059

54. Dong B (2024) A comprehensive review on toxicological mechanisms and transformation products of tebuconazole: insights on pesticide management. Sci Total Environ 908:168264
55. European Commission (2013) Ad-hoc study to support the initial establishment of the list of candidates for substitution as required in Article 80(7) of Regulation (E.C.) No 1107/2009.
56. European Commission (2015) List of Candidates for Substitution: http://ec.europa.eu/food/plant/pesticides/approval_active_substances/index_en.htm.
57. Oliveira J, Vangelie Ramos Campos E, Gonçalves M, da Silva C, Pasquoto T, Lima R, Fraceto L (2015) Solid lipid nanoparticles co-loaded with simazine and atrazine: preparation, characterization, and evaluation of herbicidal activity. J Agric Food Chem 63(2):422–432
58. Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ (2003) Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J AOAC Int 86(2):412–431
59. Lesueur C, Gartner M, Mentler A, Fuerhacker M (2008) Comparison of four extraction methods for the analysis of 24 pesticides in soil samples with gas chromatography– mass spectrometry and liquid chromatography–ion trap–mass spectrometry. Talanta 75:284–293
60. Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77:71–94
61. Stiernagle T (2006) Maintenance of *C. elegans*. WormBook 11:1–11
62. Yeates GW, Bongers T, De Goede RGM, Freckman DW, Georgieva SS (1993) Feeding habits in soil nematode families and genera - an outline for soil ecologists. J Nematol 25:315–331
63. Bongers T, Bongers M (1998) Functional diversity of nematodes. Appl Soil Ecol 10:239–251
64. Environment Canada (2005) Guidance Document on Statistical Methods (No. EPS I/RM/46). Ottawa, ON, Canada.
65. Kah M, Beulke S, Tiede K, Hofmann T (2013) Nanopesticides: state of knowledge, environmental fate, and exposure modeling. Crit Rev Environ Sci Technol 43(16):1823–1867
66. Kah M, Weniger AK, Hofmann T (2016) Impacts of (nano)formulations on the fate of an insecticide in soil and consequences for environmental exposure assessment. Environ Sci Tech 50:10960–10967
67. European Food Safety Authority (2014) Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole. EFSA J 12:98
68. Škulcová L, Chandran NN, Bielská L (2020) Chiral conazole fungicides – (Enantioselective) terrestrial bioaccumulation and aquatic toxicity. Sci Total Environ 743:140821
69. Zubrod JP, Bundschuh M, Schulz R (2010) Effects of subchronic fungicide exposure on the energy processing of Gammarus fossarum (Crustacea; Amphipoda). Ecotoxicol Environ Saf 73(7):1674–1680
70. Lefrancq M, Jadas-Hecart A, La Jeunesse I, Landry D, Payraudeau S (2017) High frequency monitoring of pesticides in runoff water to improve understanding of their transport and environmental impacts. Sci Total Environ 587:75–86
71. Man Y, Sun T, Wu C, Liu X, He M (2023) Evaluating the Impact of individual and combined toxicity of imidacloprid, cycloxaprid, and tebuconazole on *Daphnia magna*. Toxics 11(5):428
72. Eghbalinejad M, López-Cabeza R, Kotouček J, Grillo R, Koutný M, Bílková Z et al (2024) Effects of three tebuconazole nanopesticides on the survival of *Daphnia magna*. Environ Sci Nano. https://doi.org/10.1039/d3en00673e
73. Chen J, Saleem M, Wang C, Liang W, Zhang Q (2018) Individual and combined effects of herbicide tribenuron-methyl and fungicide tebuconazole on soil earthworm *Eisenia fetida*. Sci Rep 8:2967
74. Sancho E, Villarroel MJ, Fernandez C, Andreu E, Ferrando MD (2010) Short term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*). Ecotoxicol Environ Saf 73:370–376
75. U.S. Environmental Protection Agency (2023) EPISuite™ (Estimation Programs Interface Suite). [Software]. Accessed Year. https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface
76. Höss S, Roessink I, Brock TCM, Traunspurger W (2020) Response of a nematode community to the fungicide fludioxonil in sediments of outdoor freshwater microcosms compared to a single species toxicity test. Sci Total Environ 710:135627
77. Nagy K, Duca RC, Lovas S, Creta M, Scheepers PTJ, Godderis L et al (2020) Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. Environ Res 181:108926
78. Jorge-Escudero G, Pérez Polanco M, Lagerlöf JE, Pérez CA, Míguez D (2022) Commercial fungicide toxic effects on terrestrial non-target species might be underestimated when based solely on active ingredient toxicity and standard earthworm tests. Toxics 10(9):488
79. Fishel FM (2011) What are inert ingredients? PI-44. Gainesville: University of Florida Institute of Food and Agricultural Sciences.https://edis.ifas.ufl.edu/pi081. Visited January 2013.
80. Wang XX, Zhang TY, Dao GH, Hu HY (2018) Interaction between 1,2-benzisothiazol-3(2H)-one and microalgae: growth inhibition and detoxification mechanism. Aquat Toxicol 205:66–75
81. European Chemicals Agency (2021) COMPETENT AUTHORITY REPORT, 1,2-Benzisothiazol-3-(2H)-one (BIT) (PT 13) Document III-A Active Substance. https://echa.europa.eu/documents/10162/22e0994b-56c8-c3aa-4f40-65eef944114d).
82. Mueller M, Fueser H, Ngoc L, Mayer Ph, Traunspurger W, Höss S (2020) Surface-related toxicity of polystyrene beads to nematodes and the role of food availability. Environ Sci Technol 54(3):1790–1798
83. Rauchschwalbe MT, Fueser H, Traunspurger W, Höss S (2021) Bacterial consumption by nematodes is disturbed by the presence of polystyrene beads: the roles of food dilution and pharyngeal pumping. Environ Pollut 273:116471
84. Winter E, Pizzol C, Locatelli C, Crezkynski-Pasa TB (2016) Development and evaluation of lipid nanoparticles for drug delivery: study of toxicity in vitro and in vivo. J Nanosci Nanotechnol 16(2):1321–1330
85. Albuquerque FP, Oliveira JL, Dos Santos ML, Richardi VS, Navarro Da Silva MA, Pompêo MLM et al (2021) Use of non-target organism *Chironomus sancticaroli* to study the toxic effects of nanoatrazine. Ecotoxicol 30(4):733–750
86. Gomes SIL, Campos EVR, Fraceto LF, Grillo R, Scott-Fordsmand JJ, Amorim MJB (2022) High-throughput transcriptomics reveals the mechanisms of nanopesticides - nanoformulation, commercial formulation, active ingredient finding safe and sustainable-by-design (SSbD) options for the environment. Environ Sci Nano 9(6):2182–2194
87. Ayoubi M, Naserzadeh P, Hashemi MT, Reza Rostami M, Tamjid E, Tavakoli MM et al (2017) Biochemical mechanisms of dose-dependent cytotoxicity and ROS-mediated apoptosis induced by lead sulfide/graphene oxide quantum dots for potential bioimaging applications. Sci Rep 7(1):1–10
88. Del Prado-Audelo ML, Bernal-Chávez SA, Gutiérrez-Ruíz SC, Hernández-Parra H, Kerdan IG, Reyna-González J et al (2022) Stability phenomena associated with the development of polymer-based nanopesticides. Oxid Med Cell Longev 2022:5766199
89. Nevoralová M, Koutný M, Ujčić A, Starý Z, Šerá J, Vlková H, Šlouf M, Fortelný I, Kruliš Z (2020) Structure characterization and biodegradation rate of poly(ε-caprolactone)/starch blends. Front Mater 7:1–14
90. Nasser F, Lynch I (2016) Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*. J Proteomics 137:45–51
91. Mesnage R, Antoniou MN (2018) Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Front Public Health 5:1–8
92. Firdaus MAM, Agatz A, Hodson ME, Al-Khazrajy OSA, Boxall ABA (2018) Fate, uptake, and distribution of nanoencapsulated pesticides in soil–earthworm systems and implications for environmental risk assessment. Environ Toxicol 37:1420–1429
93. Zhang WP, Ruan WB, Deng YY, Gao YB (2012) Potential antagonistic effects of nine natural fatty acids against meloidogyne incognita. J Agric Food Chem 60(46):11631–11637
94. Kah M, Johnston LJ, Kookana RS, Bruce W, Haase A, Ritz V, Dinglasan J, Doak S, Garelick H, Gubala V (2021) Comprehensive framework for human health risk assessment of nanopesticides. Nat Nanotechnol 16(9):955–964

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