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Effects of three tebuconazole nanopesticides on the survival of *Daphnia magna*†

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The growing pressure to reduce excessive pesticide use has led to exploring novel formulation methods, including nanoparticle carriers for active substances. However, these emerging nanopesticides need a thorough evaluation compared to current formulations to determine whether their "nano" properties intensify toxicity for non-target organisms. This study assessed the lethal toxicity of three nanopesticides on *Daphnia magna*. The nanopesticides tested were tebuconazole loaded onto poly-ε-caprolactone, nanostructured lipid carriers, and poly(3-hydroxybutyrate) nanoparticles. Additionally, the study evaluated the toxicity of nanocarriers without loaded tebuconazole, a commercial tebuconazole formulation, and tebuconazole (pure). Effects were studied across a series of dilutions to examine the impacts on tebuconazole chemistry (total and free concentrations) and particle physics (size, heterogeneity, and density). *D. magna* exhibited varied responses to polymeric and lipid nanoformulations of tebuconazole and unloaded nanocarriers. The observed toxicity levels, from highest to lowest, were as follows: pure tebuconazole > tebuconazole-loaded on nanostructured lipid carriers > commercial tebuconazole > tebuconazole-loaded on poly(3-hydroxybutyrate) nanoparticles > tebuconazole-loaded on poly-ε-caprolactone. The mechanisms underlying the observed toxicity can generally be attributed to chemical factors (tebuconazole's free concentration and particle composition) and physical aspects (particle size and concentration). The study also revealed that dilution series can influence particle size and homogeneity, affecting the loaded substance's chemistry. This implies that the toxicity testing of nanopesticides is complex and not as straightforward as traditional dose–response modeling. Research like this is crucial to understanding the ecological impacts of nanopesticides, ensuring that new nanoformulations are beneficial rather than detrimental for plant protection.

Environmental significance

Novel formulation methods in agriculture management, including nanoparticle carriers, are being developed as a promising avenue to reduce excessive pesticide usage while maintaining efficacy. However, to recognize the potential risks of nanopesticides to non-target organisms, a comprehensive evaluation is crucial to understand their environmental impact. This study focuses on *Daphnia magna*, a key aquatic organism, to investigate the lethal toxicity of three distinct nanopesticides loaded with a widely used fungicide. The study underscores the complexity and unpredictability of nanopesticide toxicity testing, necessitating cautious interpretation of results. Insights from this research contribute to a better understanding of the ecological impact of nanopesticides and their responsible use in plant protection products.

1. Introduction

The widespread use of pesticides has raised concerns about their environmental and human health impacts, as they are commonly found in soil and aquatic environments.[1–3] One of the reasons for this indiscriminate use is the low target delivery of pesticides, as significant amounts (>90% of applied mass[4,5]) are lost due to factors such as volatilization, runoff, leaching, and degradation before they reach the target: crop pests and diseases.[6–8] To address these concerns and promote sustainable use of plant production products,

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there is a growing development of novel formulations to improve the efficiency of pesticides.

In the last decade, nanotechnology has emerged as a potential solution, with many investigations enhancing pesticide fate and efficiency and decreasing their impact on the environment and human health.[6,9,10] Nanoformulations (NFs) are generally intended to modify the environmental fate and delivery of pesticides.[6,9,11] NFs may improve the properties of agrochemicals and decrease their drawbacks, for instance, low water solubility (need for organic solvents in formulations), poor bioavailability, or fast degradation.[12,13] In fact, the intrinsic properties of nanoparticles, smaller size, and higher surface area-to-volume ratio can increase coverage on the leaves,[14] offer a greater chance to interact with the target pests and diseases, enhance the stability of the pesticides in the environment, and reduce the quantities of pesticides by increasing their efficiency.[6,9,15] Paraphrasing Ghormade *et al.* (2011),[16] instead of high-volume low-effectivity applications, nanopesticides may offer low-volume high-effectivity applications.

Polymer-based nanocarriers have received great attention in recent years due to their biocompatibility and biodegradability, which make them appropriate for different applications.[10,11,14] Synthetic polymers like poly-ε-caprolactone (PCL) and poly(3-hydroxybutyrate) (PHB) are widely used for medical applications, pharmaceuticals, packaging and cosmetic areas. Both PCL and PHB are examples of biodegradable polymers that decompose effectively in natural settings like aquatic environments, soil, and compost.[17–20] PCL has variable degradation rates in different environments, including compost and various aquatic habitats.[20] For instance, it was shown that PCL was completely assimilated over a period of six weeks in seawater and after forty-two weeks in natural fresh water.[21] Polyhydroxybutyrate (PHB) shows varying environmental degradation rates, with a half-life ranging from two months in seafloors to over three years in mesocosm pelagic tests.[17] They have attraction attention for use in the agriculture sector as well.[22,23] The modified release system of the active substance (a.s.), the high encapsulation efficiency, and the lower toxicity compared to free pesticides are confirmed as benefits of polymeric nanocarriers in different studies.[12,24–27] Lipid-based delivery systems, as another nanocarrier, are commercially known for different dosages, mostly in the pharmaceuticals sector. Low solubility and bioavailability of the a.s. are mainly two factors considered to be improved by using lipid-based NFs.[28,29] In many studies, nanostructured lipid carriers (NLC) as the second generation of lipid formulations have been prepared primarily for various therapeutic approaches[29,30] and agriculture.[31]

Nanoformulations of pesticides, while offering improvements over conventional formulations, raise significant concerns about their impact on non-target organisms.[15,32] Their higher solubility and slower dissipation could lead to increased environmental persistence and potential risks.[33] These formulations often exist as complex mixtures, posing challenges in characterization and understanding their unique physico-chemical properties. Additionally, there is a notable gap in knowledge and regulation concerning their combined risks to human health and ecosystems.[15,32] Comprehensive studies, more rigorous than those for conventional pesticides, are essential before their application in agriculture. These should consider not only chemical composition but also particle-specific properties like size, surface charge, particle distribution, number of particles, surface properties, and aggregation.[33–35] Nanomaterials introduce new methodological challenges in ecotoxicology, underscoring the need for standardized testing and robust regulations that account for their dynamic behavior.[36,37] Current legislation for plant protection products, however, does not adequately address these nanosize-specific features, particularly in environmental fate and risk assessment.[34,38] Although the active substances in pesticides are regulated within the EU, there is no specific system for testing nanopesticides.[9,15,32] Even though EFSA (2021)[39] recognized the need to evaluate nanospecific aspects (particle size, shape, structure, surface character), there are no formal guidelines yet for assessing nanoformulations of pesticides.

With the increasing number of novel nanopesticides, the scarcity of knowledge about their (eco)toxicology is alarming. However, there are some limited studies with focus on non-target organisms,[40,41] including their effects on fishes,[42] crustaceans,[42,43] algae,[43–45] terrestrial species[46] and plants.[24,31,47] In an example for non-target aquatic organisms, nanoencapsulated herbicides demonstrated increased toxicity on the survival of *Daphnia similis* after 48 hours of exposure compared to non-encapsulated herbicides. Conversely, these nanoencapsulated herbicides exhibited a lower inhibitory effect on the reproduction of *Pseudokirchneriella subcapitata* than the herbicides alone.[43] Tebuconazole (TBZ), from the conazole fungicide group, is an active substance belonging to triazole fungicides used widely in agriculture.[48] It is the 8th most used fungicide in the European Union with 1230 tonnes annually, which is 1.1% share in fungicides used in the European Union.[49] Tebuconazole treats fungal diseases in many crops, fruits, vegetables, and seeds.[50] It acts by inhibiting sterol biosynthesis in fungi (demethylation inhibitor). It has low water solubility (36 mg L^{-1} at pH 5–9, 25 °C) and a pK_α of 5.[51] TBZ is reported as a highly hazardous substance,[52] toxic to reproduction, connected to liver tumors, metabolic abnormalities, endocrine disruptor,[53,54] and altered steroid hormone levels, and affecting receptor binding. It is risky to birds and mammals and very toxic to aquatic life.[52,54,55] Moreover, it is on the EU list of candidates for substitution.[56,57] Therefore, tebuconazole was selected for this study as these mentioned limitations highlight the need to improve tebuconazole's application, increase awareness of risks, and develop alternative pest control strategies that minimize environmental impact and promote sustainable agriculture.

In our study, we aimed mainly to assess the *in vivo* toxicity of three different nanoformulations of TBZ on *Daphnia magna* immobilization (survival). Nanoformulations included poly-ε-caprolactone (PCL), nanostructured lipids (NLC), and poly (3-hydroxybutyrate) (PHB) nanocarriers (NCs) loaded with TBZ (PCL–TBZ, NLC–TBZ, and PHB–TBZ, respectively). The toxicity of nanocarriers without loaded tebuconazole and a current commercial tebuconazole formulation (FOLICUR®) was tested.

The toxicity of the compounds mentioned above was compared with pure TBZ (analytical grade). The effects were tested in dilution series to see the effects of tebuconazole chemistry (total and free concentration) and particle physics (size, heterogeneity, and concentration) on the survival of *D. magna*.

2. Materials and methods

2.1. Reagents and chemicals

Analytical standards of TBZ, PCL, Tween 80, Span 60, polyvinyl alcohol (PVA), glycerol tripalmitate (in the form of Myritol 318), and HPLC-grade acetonitrile (ACN) were purchased from Sigma Aldrich (CZ). PHB was provided by the Department of Environmental Protection Engineering (Faculty of Technology, Tomas Bata University, CZ). FOLICUR® (Bayer, 25 g TBZ L^{-1}) was purchased from local suppliers. Deionized water (dH_2O) was obtained from a Waters ultra-pure water system. The solutions were filtered through 0.22 μm Millipore nylon membrane filters. All other chemicals and solvents were of analytical grade.

2.2. Preparation of nanoformulations and nanocarriers

The following procedures were used to prepare both NFs loaded with TBZ and NCs without loaded TBZ (the same procedure but without introducing TBZ).

2.2.1. Poly-ε-caprolactone nanoparticles loaded with TBZ (PCL–TBZ). PCL–TBZ was prepared following the method of interfacial deposition of the preformed polymer with some modifications, as described by Grillo *et al.* (2012).[12] Two different phases, organic and aqueous phases, were prepared separately. To prepare the organic phase, 100 mg of PCL (polymer), 200 mg of oil (triglycerides of capric and caprylic acids, in the form of Myritol 318), 40 mg of sorbitol monostearate surfactant (span 60), and 30 mL of acetone (organic solvent) were mixed with magnetic stirring while heating at 80 °C. Later, 10 mg TBZ was added to the already cooled organic phase and stirred to mix properly. The aqueous phase constituted 60 mg polysorbate 80 surfactant (Tween 80) in 40 mL d-water. Later, the organic phase was slowly introduced to the aqueous phase while stirring. The organic phase was ultimately evaporated by a rotary evaporator. The theoretical concentration of TBZ in PCL–TBZ was 396 μg mL^{-1} in this stock solution. The chemical and physical characterizations of prepared PCL–TBZ and associated unloaded NCs are presented in Table 1.

2.2.2. Nanostructured lipid carriers loaded with TBZ (NLC–TBZ). NLC–TBZ was prepared following the emulsification method with solvent evaporation described by De Oliveira (2015)[31] with some modifications. Briefly, 250 mg of glyceryl tripalmitate, 75 mg of Myritol, and 10 mg of TBZ were dissolved in 5 mL of chloroform in a covered beaker to avoid solvent losses. This organic phase (lipid phase) was introduced to an aqueous phase, constituting a solution with 370 mg of PVA in 30 mL of Milli-Q water. The mixture was sonicated for 4 min at 100% power in an ice bath. Ultimately, the pre-emulsion was homogenized by a Turrax at level 5 for 10 min. The organic phase was then evaporated under rotary evaporation to get 20 mL emulsion. The theoretical concentration of TBZ in NLC–TBZ was 381 μg mL^{-1} in this stock solution. The chemical and physical characterizations of prepared NLC–TBZ are presented in Table 1.

2.2.3. Poly 3-hydroxybutyrate nanospheres loaded with TBZ (PHB–TBZ). PHB was prepared following the solvent/evaporation technique, according to the method used by Salač *et al.* (2019)[58] and López-Cabeza *et al.* (2022).[13] Briefly, the organic phase was prepared by dissolving 300 mg of PHB in 10 mL of chloroform with reflux heating at 130 °C on magnetic stirring. TBZ (90 mg) was subsequently added to the organic phase. This organic phase was then inserted into an aqueous phase (100 mL) containing 0.5% (w/v) PVA, and the mixture was emulsified by a higher-speed homogenizer (14 000 rotations per min for 5 min). The emulsified mixture was homogenized by an ultrasonic probe SOOPULS HS 20 with amplitudes of 50% (for 5 min). The organic phase was ultimately evaporated with gentle stirring to the final volume of 100 mL. The theoretical concentration of TBZ was 900 μg mL^{-1} in this stock solution. The chemical and physical characterizations of prepared PHB–TBZ are presented in Table 1.

2.3. Physical characterization of nanoformulations and nanocarriers

Particle diameter (hydrodynamic diameter (HDD)), polydispersity index (PI), particle concentration, and zeta (ζ) potential were

Table 1 Total concentration of tebuconazole (TBZ), hydrodynamic diameter (HDD), polydispersity index (PI), ζ potential, particle concentration, and encapsulation efficiency (EE) of the nanoformulations of TBZ loaded on poly-ε-caprolactone (PCL), nanostructured lipid carrier (NLC) and poly(3-hydroxybutyrate) (PHB) NFs and the corresponding nanocarriers unloaded with TBZ (NCs). The values are the mean ± standard deviation (n = 3)

Nanoformulation/nanocarrier	Total TBZ concentration (μg mL^{-1})	HDD (nm)	PI	ζ potential (mV)	Particle concentration (particles per mL)	EE (%)
PCL–TBZ	314.0 ± 5	226 ± 3[a]	0.195 ± 0.02[a]	−37.5 ± 0.5	4.1×10^{11}	96.9 ± 2.3
PCL	—	223 ± 2[a]	0.162 ± 0.01[a]	−25.6 ± 0.6	2.7×10^{11}	—
NLC–TBZ	313.5 ± 11	340 ± 10[a]	0.218 ± 0.02[a]	−37.9 ± 0.4	1.8×10^{11}	92.0 ± 5.0
NLC	—	300 ± 4[a]	0.192 ± 0.01[a]	−32.7 ± 0.4	3.9×10^{11}	—
PHB–TBZ	224.0 ± 11	269 ± 4[a]	0.193 ± 0.04[a]	−37.8 ± 0.3	2.3×10^{9}	83.7 ± 0.4
PHB	—	275 ± 5[a]	0.250 ± 0.01[a]	−37.5 ± 0.4	4.7×10^{10}	—

[a] Indicates no significance to each NF with its unloaded NC value ($p \leq 0.05$).

measured to assess the stability of the suspensions of NCs in amber flasks at room temperature (25 °C).

For the determination of the size and concentration characteristic, approximately 60 μL of the suspension of NFs and NCs was placed in a low-volume quartz batch cuvette ZEN2112 (Malvern Panalytical Ltd., Malvern, UK) and measured by a Zetasizer Ultra (Malvern Panalytical Ltd., UK) using the multi-angled dynamic light scattering technique (MADLS®), at a constant temperature of 25 °C. The device was equipped with a HeNe Laser (633 nm) and three detectors at angles: 173° (backscatter), 90° (side scatter), and 13° (forward scatter). The measured data were evaluated using ZS Xplorer software version 1.50 (Malvern Panalytical Ltd., UK). The measured values of hydrodynamic diameter, polydispersity index (PI), and particle concentration are reported as mean ± standard deviation (n = 3).

The ζ potential measurements were performed on a ZetaSizer Ultra (Malvern Panalytical Ltd., UK) using the Electrophoretic Light Scattering (ELS) method. Approximately 800 μL of the suspension of NFs and NCs was placed in disposable folded capillary cells DTS1070 (Malvern Panalytical Ltd., Malvern, UK) and measured at a constant temperature of 25 °C using automatic mode. The ζ-potential values were calculated using the Smoluchowski equation and are reported as mean ± standard deviation (n = 3).

2.3.1. Nanoparticles' morphology (transmission electron microscopy). A drop of the suspension was placed on a grid coated with Formvar (Sigma-Aldrich, Czech Republic) and carbon (Agar Scientific, Austria). After approximately 1 minute, the residual water was dried with the use of filtration paper. The resulting sample was observed using a TEM Philips 208 S Morgagni (FEI, Czech Republic) at 7500× magnification and an accelerating voltage of 80 kV.

2.4. Chemical characterization of nanoformulations

The total concentration of TBZ in all three NFs was measured after extraction with ACN. In this regard, 200 μL of each NF was added to 19.8 mL acetonitrile, and the mixture was mixed for 30 s by a vortex mixer. Then, the extract was diluted ten times (n = 3), filtered through nylon filters (0.22 μm), and analyzed by HPLC-MS/MS as described in detail in López-Cabeza *et al.* (2022).[13]

Encapsulation efficiency (EE%), as the percentage of TBZ loaded in each NC, was calculated using the following equation:

$$\mathrm{EE}\ (\%) = \frac{m_{\mathrm{loaded}}}{m_{\mathrm{total}}} = \frac{m_{\mathrm{total}} - m_{\mathrm{free}}}{m_{\mathrm{total}}} \quad (1)$$

Here, m_{loaded} is the mass of TBZ loaded on NCs, m_{total} is the total mass of TBZ calculated from the total concentration, and m_{free} is the amount of TBZ that is not associated with NCs which was determined by the ultrafiltration–centrifugation method as follows: 0.5 mL of each NF was placed in a 30 kD regenerated cellulose filter (Microcon®, Millipore) and centrifuged for 30 minutes at 14 000 × g (n = 3). The filtrates after dilution (1 : 100) were analyzed by HPLC-MS/MS as described in detail in López-Cabeza *et al.* (2022).[13]

2.5. Toxicity testing

2.5.1. *Daphnia magna* cultures. *D. magna* was cultured in 6 L beakers filled with ADaM[59] (pH = 7.7–8.4, details of ADaM compositions in Table S1†) at a constant temperature of 20 ± 2 °C with 16 : 8 h (light : dark) photoperiod and fed three times per week with 2 mL of live green alga suspension containing *Desmodesmus subspicatus* in 10^9 cells per mL concentration, cultivated in our laboratory. For maintaining *D. magna*, 1/3 of the old medium was thrown away and replaced with a newly aerated medium once a week.

2.5.2. Immobilization test. Acute immobilization tests (48 h) were carried out according to OECD guidelines for testing chemicals 202 (2004)[60] on *Daphnia* sp. Despite the original testing procedure with *D. magna* involving vessels made of plastics, in our experiments, 50 mL glass beakers were utilized as test vessels to avoid any sorption to the test containers. In all the experiments, the beakers were filled with 20 mL test solutions. Then, seven neonates, <24 h old in each test vessel in four replicates, were exposed to different NFs (PCL–TBZ, NLC–TBZ, and PHB–TBZ), their associated unloaded NCs (PCL, NLC and PHB), FOLICUR® and pure TBZ separately in ADaM medium under static conditions (without refreshing the exposure medium, and no feeding) at 20 ± 2 °C, 16 : 8 (light : dark) photoperiod. Based on OECD 202 guidelines,[60] daphnid immobilization is recorded visually at 48 h, and the EC_{50}, which is the concentration expected to immobilize 50 percent of daphnids in a tested exposure medium, was determined. Daphnids that were able to swim freely or after 15 seconds of gentle agitation were considered as survived (those with moving their antennae were considered immobile).

At first, a range-finding experiment was performed to find the effective dose of pure TBZ. Considering the solubility limit of TBZ in water (36 mg L^{-1}, 20 °C), the experimental setup was designed based on the concentrations below the TBZ solubility limit. However, the maximum concentration that was possible to dissolve homogeneously in ADaM medium was 13 mg L^{-1}, as verified by LC-MS/MS. Then dilution series were prepared for the range-finding test with nominal concentrations of 0.26, 0.54, 1.49, 2.47, 4.67, 7.50, and 13 mg L^{-1}. Based on the result of this range-finding test (TBZ EC_{50}= 6.7 mg L^{-1}), five concentrations of TBZ for each tested compound were selected for the experiments. The final test nominal concentrations included: 2.25 (C1), 4.5 (C2), 9 (C3), 18 (C4), and 36 (C5) mg L^{-1} (all concentrations are expressed as mg TBZ in L of ADaM medium). As will be shown later, the real concentrations (measured by HPLC-MS/MS for each compound in the test medium) differed from the calculated nominal concentrations and were different for different compounds tested. Thus, the universal C1–C5 coding is used for clarity.

The tested concentrations were prepared by serial dilution from the highest concentration (C5), which was prepared by adding an aliquot of NFs stock suspension, commercial FOLICUR®, or pure TBZ stock solution to fresh aerated ADaM medium. Then, C4 was prepared from C5 by dilution with factor of 2. The rest of the concentration was prepared from the lower

dilution treatment. At the same time, another exposure scenario was done in which the effect of NCs (without TBZ) on the immobilization of *D. magna* was assessed. NCs' solution (without TBZ) was prepared based on the dilution factor used for its counterpart NCs with TBZ.

The total concentrations and the concentration of free TBZ for all treatments were verified at t = 0 and t = 48 by HPLC-MS/MS (see in section 2.4). Likewise, the physical properties of NFs and their NCs, including HDD, PI, and particle concentration, were verified by MADLS (ZetaSizer Ultra, Malvern Panalytical Ltd, UK) at t = 0 and t = 48 h.

Acute toxicity tests with potassium dichromate ($K_2Cr_2O_7$) as a reference substance were carried out every month to check the sensitivity of the *D. magna* culture whose EC_{50} was between 0.6 and 1 mg L^{-1} corresponding to the test validity criteria defined by OECD (2004).[60] Test media were checked at the beginning and the end of the test for pH and dissolved oxygen using a YSI multi-lab 4010-1 W water quality instrument. The dissolved oxygen concentration was always >3 mg L^{-1} in the culturing beaker and test medium (=8 mg L^{-1}). pH was between 7.4 and 8.4.

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 used before analysis. If the assumptions were not validated, data were log-transformed. The concentration of TBZ was calculated for the mean concentration of the measured concentrations at t = 0 and t = 48 h, and the data are expressed as the mean with the corresponding standard deviation (SD) (n = 2). The survival data was calculated based on the mean of surviving daphnids after 48 h exposure (n = 4). The percentage of survivals was calculated based on each vessel's surviving daphnids divided by the mean number of surviving adults in the control (without any added substance) and multiplied by 100. Survival data were analyzed by GraphPad Prism 9.5.1(GraphPad Software, USA). The concentration–response relationship with 95% confidence limit was modeled by nonlinear logarithmic regression based on the Hill equation called "log(agonist) *vs.* response (Find EC anything" in the GraphPad Prism library). The regression fit was based on the least squares. This four-parameter logistic fit equation was employed to determine the effective concentration at which 50% (EC_{50}) of the neonates were affected. Dose–response curves were drawn from GraphPad Prism 9.5.1(GraphPad Software, USA), and the rest of the graphs were drawn in Excel (MS Office). The comparison of the endpoint for (nano)formulations with pure TBZ was performed by the two sample two-tailed T-test according to the F-test method.[61–64]

3. Results and discussion

3.1. Characterization of the nanoformulations and nanocarriers

The stock suspensions of the prepared nanoformulations (NFs) loaded with tebuconazole (TBZ) and the corresponding nanocarriers (NCs) unloaded with TBZ were characterized by measuring the total concentration of TBZ, hydrodynamic diameter (HDD), polydispersity index (PI), ζ potential and particle concentration (Table 1). Total TBZ concentration was relatively similar in PCL–TBZ and NLC–TBZ but significantly lower in PHB–TBZ compared to their theoretical (expected) concentrations. HDD was lower in PCL particles, followed by PHB particles and the largest NLC particles. The particle size of NFs loaded with TBZ was similar to that of their corresponding unloaded NCs in the case of PCL and PHB, while for NLC, unloaded particles were smaller by 12% than loaded NFs. PI was in the range of 0.160–0.250. ζ potential was in the range of −37 to −38 mV, except for lower values of unloaded NLC and PCL. The highest concentration of particles was found for PCL, followed by NLC, and then by 1–2 orders of magnitude lower concentrations of PHB. Unloaded NCs had higher concentrations than corresponding loaded NFs in the case of PHB and NLC, but it was reverse for PCL.

Total TBZ concentration in PCL–TBZ and NLC–TBZ was only slightly lower than expected from the preparation (386 and 391 mg L^{-1}, respectively). Still, in the case of PHB–TBZ, the measured TBZ concentration was only 25% of the expected one (224 *versus* 900 mg L^{-1}). The total concentration of TBZ in PHB–TBZ was theoretically 900 μg mL^{-1}. TBZ degradation is not probable within the time between NF preparation and measurement, as TBZ is considered very slowly degrading.[51] It seems that PHB–TBZ showed resistance to ACN, which was used to extract TBZ from the NFs. Perhaps the ACN used in our experiment was not strong enough to release TBZ from the PHB polymer. Other solvents like methanol or chloroform may bring higher extraction efficiency.

There is still debate about the size of nanoparticles in nanopesticides. According to the literature, the nano-range refers to particle sizes in hundreds of nm.[6,34,65] Therefore, HDD results show that the NFs and NCs in our study are typical nanopesticides and nanoparticles, respectively. The physical properties of NFs are similar to previous studies where the same carriers were used (PCL,[12] NLC,[31] and PHB[13]). The PI shows the HDD distribution of the particles and, thus, the homogeneity of the particle population. PI < 0.2 is considered ideally homogenized for polymeric and lipid nanoparticles.[12] All synthesized NCs and NFs showed PI values around 0.2, indicating good particle homogeneity. ζ potential shows the charges on the particle surfaces and indicates the nanoparticle stability. ζ potential with approximately (±)30 mV shows stable nanoparticles. The negative values indicate the presence of carboxylic acid groups.[12,31]

EE is a parameter showing the association of an active substance (a.s.) and NC. Various factors, such as the specific chemical properties of the substance and the surface characteristics of the nanoparticles, can influence the binding affinity. A higher surface area and smaller nanoparticle size allow the a.s. to be encapsulated in the nanoparticle. On the other hand, the chemical properties and polarity of the a.s. increase the association efficiency in lipid and polymeric nanocapsules.[12,13] In this study, EE% values were between 84

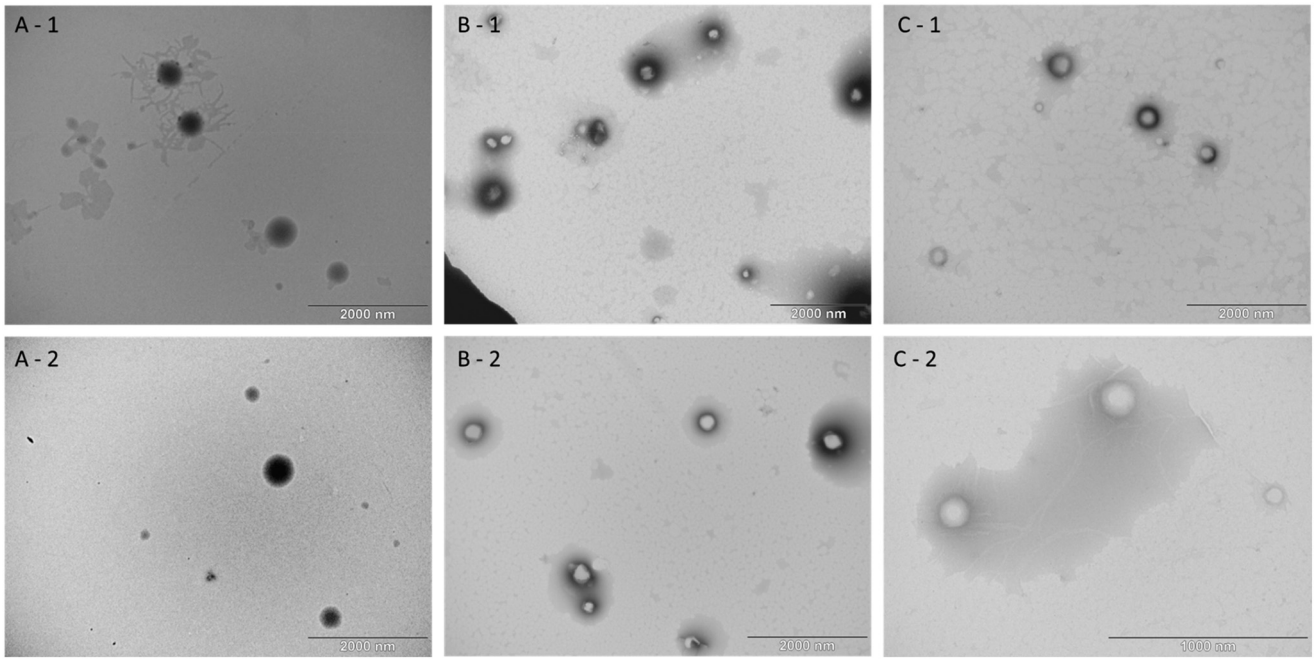

Fig. 1 Transmission electron microscopy micrographs of nanoparticles, A) poly-ε-caprolactone (PCL), B) nanostructured lipid carriers (NLC), and C) poly(3-hydroxybutyrate) (PHB) particles. Within each subfigure, index 1 denotes particles unloaded with TBZ, while index 2 represents particles loaded with TBZ.

and 97%, demonstrating the hydrophobic nature of all NCs with a tremendous binding affinity for TBZ and good association efficiency of all tested NFs.

3.1.1. Nanoparticles' morphology (transmission electron microscopy). Transmission electron microscopy (TEM) micrographs, illustrated in Fig. 1, showcase the morphological features of various nanoformulations, including A) NLC, B) PCL, and C) PHB particles. Within each subfigure, index 1 denotes particles unloaded with TBZ, while index 2 represents particles loaded with TBZ. The focus of this analysis is to elucidate the uniformity of the particles and the absence of aggregation, particularly in the context of TBZ loading. A notable alignment between TEM analysis and DLS results is observed, substantiating the reliability of our findings. This correspondence reinforces confidence in the reported morphological and dimensional attributes of TBZ-loaded nanoparticles. Additionally, the application of both TEM and DLS contributes to a comprehensive understanding of the particulate system.

3.2. Fate and behavior of TBZ after dilution in the exposure medium

Table 2 shows the total and free concentrations of TBZ measured in exposure medium with the different treatments: PCL–TBZ, NLC–TBZ, PHB–TBZ FOLICUL®, and pure TBZ. The data are the mean of concentrations at t = 0 and t = 48 h (n = 2). Details of concentrations at t = 0 and t = 48 h are separately provided in S2–S4.†

In more diluted treatments (C1–C3), the total and free TBZ concentrations were relatively similar in all three NFs. In comparison, in lower dilution treatments (C4–C5), the total TBZ concentration was significantly higher than the free TBZ. The relative proportion of free concentration to the total concentration increased with an increase in the dilution. It may be the result of the solubility limit of TBZ. In lower dilution treatments, the total concentrations are near the solubility limit of TBZ (solubility of TBZ = 36 mg L^{-1}).

Table 2 The total and free concentration (mg L^{-1}) of tebuconazole (TBZ) in different (nano)formulations: poly-ε-caprolactone (PCL), nanostructured lipid carrier (NLC), poly(3-hydroxybutyrate) (PHB), commercial formulation (FOLICUR®) and pure TBZ, after dilution in ADaM medium *versus* nominal (theoretical) concentration of TBZ. The concentrations are the mean of the measured concentrations of TBZ at t = 0, t = 48 h (each time n = 3). The concentration of TBZ for NFs is presented for the total concentration of loaded TBZ (TC) and free TBZ. The values (in this table) are the mean ± standard deviation (n = 2)

	Nominal concentration	PCL–TBZ		NLC–TBZ		PHB–TBZ			
		Total	Free	Total	Free	Total	Free	FOLICUR®	Pure TBZ
C1	2.25	2.32 ± 0.14	2.10 ± 0.06	2.24 ± 0.05	2.27 ± 0.04	2.65 ± 0.28	2.28 ± 0.15	2.28 ± 0.10	1.10 ± 0.10
C2	4.50	4.13 ± 0.14	3.58 ± 0.33	3.89 ± 0.05	3.96 ± 0.03	6.90 ± 0.23	5.09 ± 0.15	4.75 ± 0.10	2.30 ± 0.10
C3	9.00	8.67 ± 0.35	6.21 ± 0.17	8.32 ± 0.50	7.72 ± 0.06	13.42 ± 1.57	10.55 ± 0.65	8.76 ± 0.10	3.84 ± 0.10
C4	18.00	18.02 ± 0.50	7.98 ± 0.42	16.98 ± 0.60	13.38 ± 0.64	29.13 ± 4.18	17.20 ± 0.45	14.00 ± 0.30	7.16 ± 0.30
C5	36.00	34.19 ± 1.33	10.21 ± 0.41	32.50 ± 1.05	20.34 ± 0.68	121.48 ± 34.00	24.60 ± 1.10	32.32 ± 1.70	13.30 ± 0.70

Relatively similar values for total and free TBZ concentrations with increasing dilution reveal that the association with NCs could not modify the release rate of TBZ in exposure media (*e.g.*, bringing slow-release feature), which can be explained by two aspects: 1) as a result of the lower affinity of TBZ to NCs, and also, 2) decrease of the stabilization agent concentration (*e.g.*, PVA) in higher dilutions.

The difference between the total and free concentration of the a.s. in nanopesticides can illustrate their durability, an essential factor to consider in their regulatory evaluation and environmental sustainability. The durability of nanopesticides refers to their ability to remain effective over time and under different environmental conditions.[9,34] The release rate of the a.s. provides essential information about the interaction of the a.s. and the carrier.[25,31] Thus, the higher affinity of the a.s. to the NCs would result in the lower and slower release of the a.s. Carrier properties, distribution, loading, and solubility of the a.s. by diffusion and/or dissolution can control the release mechanism.[11]

Moreover, as stabilization agents play a role in the release of the a.s, it is needed to state that stabilization agents are critical in the formulation and stability of NFs, commonly used to prevent the agglomeration of nanoparticles and to maintain their stability in solution.[66] They can improve the stability, particle size, mobility, and efficacy of nanoparticles, which is essential for their use in various applications such as drug delivery, environmental remediation, and agriculture.[67] Surfactants due to their amphiphilic nature are exploited to stabilize hydrophobic nanomaterials in aqueous media. Surfactants like PVA and polysorbate (*e.g.*, Tween-80) are highly effective as colloidal stabilizers of suspensions used for decades in the synthesis of different nanomaterials, especially in polymeric nanocarriers, benefited from their high biodegradability in the environment.[12,31,66,68,69] Tween-80 as a non-ionic surfactant consisting of polyethoxylated sorbitan and oleic acid is often used also in food products due to its lower toxicity and high emulsifying ability,[70] while the simultaneous use of Tween-80 with high hydrophilic surfactants, for instance span surfactants, in the fabrication of nanomaterials can significantly improve the hydrophobicity of nanomaterials.[71]

Similar to our result in PCL–TBZ in a study by Grillo *et al.* (2012),[12] the release of three encapsulated herbicides from PCL (in one concentration) was investigated in water. Results showed that the encapsulation method could significantly improve the release of herbicide. The degree of herbicides' hydrophobicity was mentioned as an essential factor in the release behavior, in addition to the interaction of the compound with the polymeric chains. The relaxation of the polymer chains was reported as a mechanism of release.[12] Interestingly, the release of herbicide from PCL nanoparticles in the soil matrix is much lower than that in water due to the absence of diffusive media; thus, the environment is also an essential factor to be evaluated.[72] Moreover, in the confirmation of our study with the PHB carrier, the modified release of the a.s. from the PHB carrier was reported in other studies.[13,22,73]

In a study by De Oliveira *et al.* (2015),[31] solid lipid nanoparticles of TBZ showed a modified release of the active substance. De Oliveira (2015)[31] prepared solid lipid nanoformulations loaded with different herbicides. The release kinetics demonstrated that solid lipid NCs modified the release profiles in water. The significantly slower release of herbicides (one concentration) in NFs was reported compared to free herbicides.[31]

3.3. Physical characterization of nanoformulations and nanocarriers after dilution in the exposure medium

Insight into possible changes in NC properties compared to their stock suspension is essential as it is predicted that NCs' physical properties in facing real environments will change.[32,35] To understand the fate and behavior of NCs with the exposure conditions, it is crucial to assess the physicochemical properties of NCs in the exposure media.[74]

Plots A, B, and C in Fig. 2 show the physical properties of PCL–TBZ and PCL NC unloaded with TBZ in the exposure medium. The mean HDD and PI gradually decreased significantly with increasing dilution. Particle concentration decreased with increasing dilution. However, the decrease in the number of particles did not follow the dilution factor precisely (here, the dilution factor was 2). The behavior of PCL nanoparticles loaded and unloaded with TBZ seemed very similar except for the lowest dilution (C5), where the concentration of particles was significantly lower in the case of PCL–TBZ. This was surprising because, in stock, PCL–TBZ had a higher particle concentration than PCL without TBZ.

Plots D, E, and F in Fig. 2 show the physical properties of NLC–TBZ and NLC unloaded with TBZ in the exposure medium. The mean HDD was bigger than the stock suspension (Fig. 2, plot D and Table 1). With the decrease in dilution, NLC NCs in the tested concentrations tended to increase the HDD slightly.

The PI seemed to increase with decreasing dilution, which is evidence of starting aggregation. Some factors may affect the physical properties of NCs in exposure medium, *e.g.*, exposure medium's properties like pH and ionic strength.[35] In the present study, NLC treatments were acidic (pH is not provided here), which may relate to the changes in physical properties of exposure medium with NLC.

Although with high variability, the lowest and highest dilutions seem to have lower particle concentrations than the C2–C4 dilutions. High variability of the NLC particle concentration demonstrates that there were some issues in MADLS measurements. MADLS showed errors in data quality in higher dilutions. MADLS measures can be biased toward larger particles as the smaller particles block detection in a heterogeneous sample.[35] It is assumed that the dilution in the ADaM medium and its properties like pH (acidic in NLC suspension) resulted in heterogeneity in the NLC treatments. Similar to PCL, the particle concentration in NLC did not follow the dilution factor precisely.

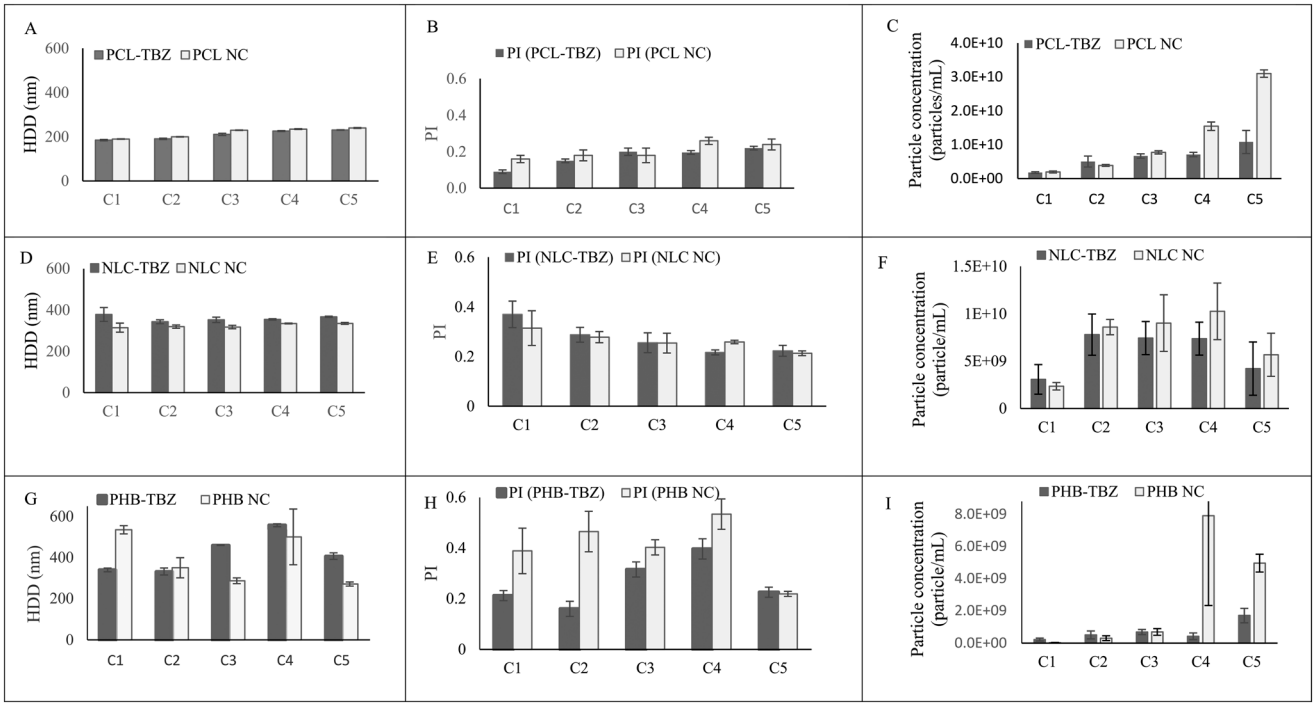

Fig. 2 The physical properties of tested nanoformulations (NFs) loaded with tebuconazole (TBZ) and the corresponding nanocarriers (NCs) unloaded with TBZ in the exposure medium over 48 h. The plots in the same column show the same parameters: hydrodynamic diameter (HDD) – plots A, D, G, polydispersity index (PI) – plots B, E, H, and particle concentration – plots C, F, I. The plots in the same row show the results for the same carrier: poly-ε-caprolactone (PCL) – plots A–C, nanostructured lipid carriers (NLC) – plots D–F, and poly(3-hydroxybutyrate) (PHB) – plots G–I. The values show the mean ± SD (n = 3).

Plots G, H, and I in Fig. 2 show the physical properties of PHB–TBZ and PHB unloaded with TBZ in exposure medium. Compared to PCL and NLC, PHB showed a less consistent pattern of all physical parameters. With the increase in dilution, HDD increased for PHB NC unloaded with TBZ (except for C4, which also has very high variability). For PHB–TBZ, the particle size increased in C4 but decreased with increasing dilution. PI showed increased heterogeneity in particle population from C4 and higher dilutions, especially for PHB unloaded with TBZ (which also suffered from very high variability). It should be mentioned that MADLS showed an error in data quality for higher dilutions. These values of particle concentration cannot be seriously considered.

The physics of PHB was seemingly affected in exposure medium by dilution. It can be assumed that changes in physics, *e.g.*, the size of PHB in higher dilution treatments, result from decreasing the concentration of stabilization agents used in the composition of PHB (for PHB, PVA was used as a stabilization agent).

3.4. Toxicity for *Daphnia magna*

D. magna is a vital member of the aquatic trophic webs as any changes in the quality and quantity of their population can affect other aquatic organisms. Thus, using this invertebrate is relevant as a bioindicator to assess the toxicity effect of chemicals in the environment. It is also recommended as a test organism in several international test guidelines.[60,75] *D. magna* has also been used for years to study nanotoxicity. Their results are instrumental in advancing our understanding of the potential risks and impacts of nanomaterials on aquatic organisms.[32,76–78]

The growing interest in using NFs of agrochemicals led us to assess the toxicity of different nanomaterials, polymeric, and lipid-based nanomaterials loading tebuconazole on the survival of *D. magna*.

The results of acute *D. magna* immobilization toxicity tests of NFs loaded with TBZ and NCs unloaded with TBZ are presented in Table 3 and Fig. 3, together with the results of the commercial formulation FOLICUR® and analytical grade TBZ. Table 3 shows the TBZ concentrations in different (nano) formulations causing 50% (EC_{50}) effects on the survival of *D. magna*. The endpoint is reported based on the total concentration of TBZ and free concentration of TBZ. EC_{50} based on the total TBZ concentration was significantly higher (*i.e.*, the toxicity was lower) for PCL–TBZ, PHB–TBZ, and FOLICUR® in comparison with NLC–TBZ and pure TBZ ($p < 0.05$). Generally, the order from the most to the least toxic formulation, based on the EC_{50} values, was: pure TBZ > NLC–TBZ > FOLICUR® > PHB–TBZ> PCL–TBZ.

3.4.1. PCL–TBZ. Plot A in Fig. 3 shows the effects on survival of *D. magna* for PCL–TBZ (based on the total TBZ concentration and free TBZ concentration) compared with pure TBZ. The survival decreased with increased TBZ concentration within the tested concentration range. In lower concentrations of TBZ, the survival (%) of daphnids between PCL–TBZ and pure TBZ was

Table 3 Effective concentrations of tebuconazole (TBZ) in different (nano)formulations of TBZ: poly-ε-caprolactone (PCL), nanostructured lipid carrier (NLC), poly(3-hydroxybutyrate) (PHB), commercial formulation (FOLICUR®), and pure TBZ. The values show concentrations of TBZ (in mg L^{-1}) causing 50% mortality (immobilization) of *Daphnia magna* EC_{50}). The values for nanoformulations are expressed for both total and free concentrations of TBZ. In the parentheses, 95% confidence intervals are shown for the effective concentrations

	Tested compounds (TBZ formulations, mg L^{-1})							
	PCL–TBZ		NLC–TBZ		PHB–TBZ			
	Total	Free	Total	Free	Total	Free	FOLICUR®	Pure TBZ
EC_{50}	12.3 (9.0–16.8)[a]	6.5 (5.4–7.7)[a]	6.5 (5.0–8.3)[a]	6.1 (5.0–7.7)[a]	11.4 (8.5–14.2)[a]	7.5 (5.5–10.0)[a]	9.6 (8.1–11.1)[a]	6.7 (5.2–8.2)

[a] Indicates significant and no significant differences, respectively, from pure TBZ ($p \leq 0.05$).

similar. With the increase in TBZ concentration, the difference between PCL–TBZ and pure TBZ became more remarkable. Also, the toxicity expressed based on free TBZ concentration was similar to the pure TBZ curve. Based on the results of chemical analyses (Table 2), PCL–TBZ showed a decline in toxicity caused by the decrease in bioavailability of TBZ (free TBZ concentration). The generally low toxicity of PCL-based nanopesticides has been reported in several studies, and therefore, PCL has been recommended as a suitable carrier for drugs.[74,78] In contrast to the toxicity reduction caused by the nanoformulation observed in our study, Clemente *et al.* (2013)[43] reported that ametrine and atrazine encapsulated in PCL showed significantly greater toxicity to *Daphnia similes* after 24 and 48 h in comparison with the pure a.s. The toxicity of NFs of atrazine increased over 48 h and was attributed to the increase in bioavailability of atrazine. The combined effect of atrazine

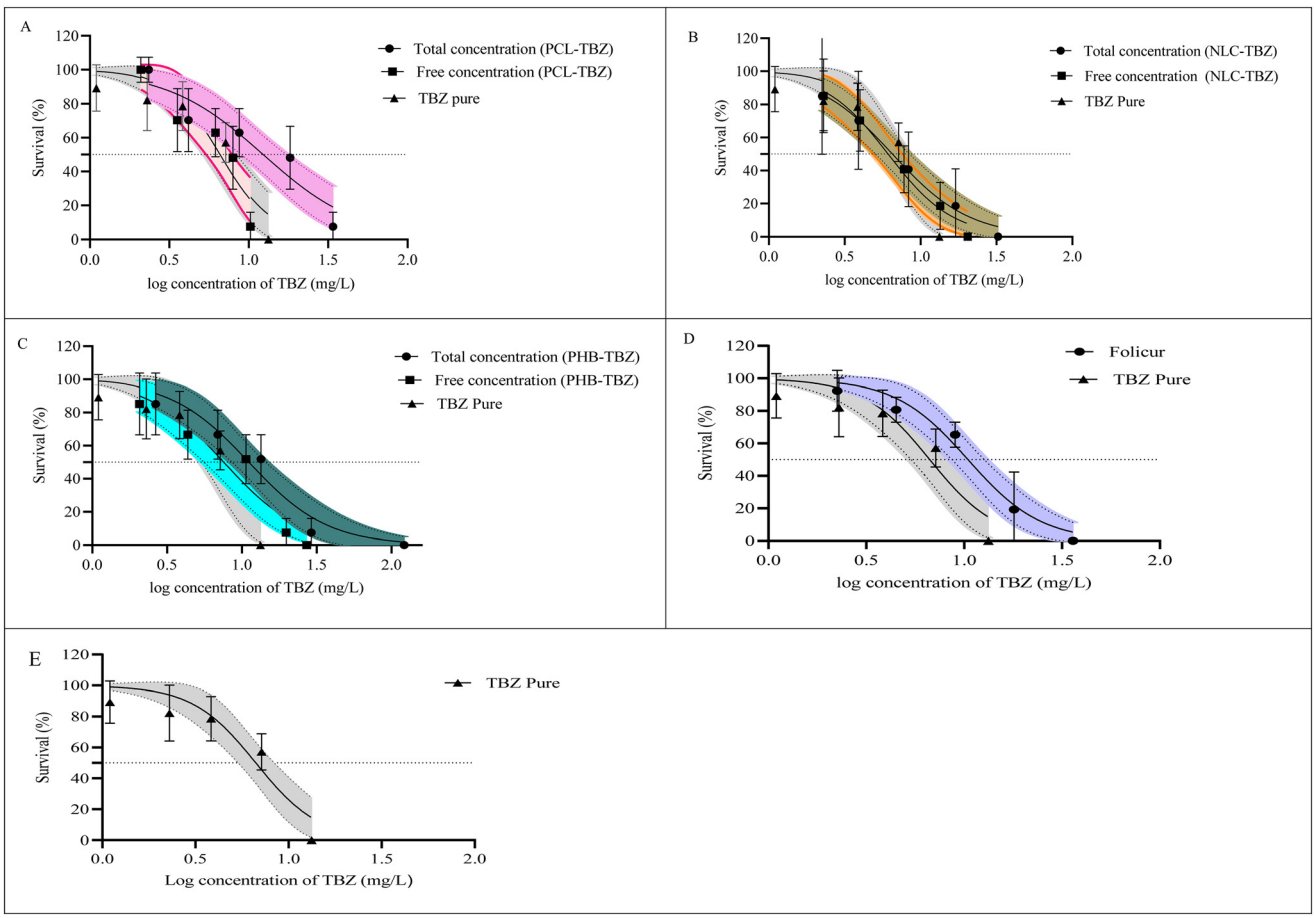

Fig. 3 Survival of *Daphnia magna* neonates in response to 48 h exposure to different concentrations of tebuconazole (TBZ) in different (nano) formulations: A) poly-ε-caprolactone (PCL), B) nanostructured lipid carrier (NLC), C) poly(3-hydroxybutyrate) (PHB), D) commercial formulation (FOLICUR®), and E) pure TBZ. For each type of formulation, the results are presented together with the effects of pure TBZ. For the nanoformulations (NFs), the results are expressed based on both the total and free concentration of TBZ. Color bands show 95% confidence intervals around the non-linear regression. The non-linear regression is based on the Hill equation called log(agonist) *vs.* response in GraphPad Prism and the least squares method. The measured toxicity results in each concentration are shown as mean + 95% CI (n = 4).

with other compounds in the NFs, such as co-polymers, was also credited as the toxicity of NFs to *Daphnia similes*.[43] An EC50 of 204 mg L^{-1} has been reported for *D. magna* exposed to epsilon-caprolactone (ε-caprolactone),[43] which is higher than the concentration of PCL used in the present study. However, free components such as surfactants may adversely affect the survival of *D. magna*. According to some studies, polysorbate 80 (Tween-80) is non-toxic to *D. magna*,[79] unlike triglycerides of capric and caprylic acids (Myritol 318). The reported EC_{50} for caprylic acids varies between 17 and 46 mg L^{-1}.[43] It is suggested that the metabolization of this compound could result in the release of this compound to the medium and induce toxicity.[43] In the present study, the concentration of caprylic acids (in the form of Myritol 318 here) in the exposure medium was higher than the above-reported EC_{50}, which can be a reason for PCL–TBZ toxicity to *D. magna*. There is no data regarding the toxicity of sorbitan monostearate to daphnids.[43]

3.4.2. NLC–TBZ. Plot B in Fig. 3 shows the effects on survival of *D. magna* for NLC–TBZ (based on the total TBZ concentration and free TBZ concentration) compared with pure TBZ. The survival decreased with increased TBZ concentration within the tested concentration range. The survival (%) of daphnids between NLC–TBZ (based on total and free concentrations) and pure TBZ was similar among the tested concentrations of TBZ. Based on the results of chemical analyses (Table 2), NLC–TBZ showed a higher release of TBZ at higher total TBZ concentrations, which increased the toxicity caused by increased bioavailability of TBZ (free TBZ concentration).

It might be possible that lipid-based nanopesticides do not significantly alter the toxicity of the a.s. Albuquerque *et al.* (2021)[80] tested solid lipid nanoparticles loaded with atrazine on the non-target organism, *Chironomus sancticaroli*, and they reported lethal effects at 2 μg L^{-1} atrazine (as an environmentally relevant concentration). Moreover, the nanoparticles loaded with atrazine showed similar toxicity to free atrazine which led to mortality and biochemical changes in *Chironomus* larvae.[80] Similarly, in the present study, NLC–TBZ showed similar toxicity to free TBZ (Fig. 3B and Table 3) on the survival of *D. magna*. In the case of NLC, the effect of TBZ concentration (as the concentration increases) plays a dominant role in the survival of daphnids compared to particle concentration and HDD. However, the presence of Myritol 318 in the composition of NLC could also lead to toxicity on the survival of daphnids. In the present study, the concentration of Myritol 318 in the exposure medium within the tested concentrations of NLC–TBZ was higher than reported for EC_{50} on *D. magna* exposure to Myritol 318 (explained in the 3.5 PCL section). The effect of particle concentration and HDD will be presented in the following section (3.5.2).

3.4.3. PHB–TBZ. Plot C in Fig. 3 shows the effects on survival of *D. magna* for PHB–TBZ (based on the total TBZ concentration and free TBZ concentration) compared with pure TBZ. The survival decreased with increased TBZ concentration within the tested concentration range. At lower concentrations of TBZ, PHB–TBZ (based on both total and free TBZ concentration) showed similar toxicity to *D. magna* to pure TBZ, indicating that TBZ bioavailability is not strongly affected by the nanoformulation. However, at higher concentrations, the toxicity significantly decreased compared to pure TBZ ($p < 0.05$) (Fig. 3C and Table 3). This could be explained by the lower bioavailability of TBZ in higher concentrations of PHB–TBZ, which would lead to more survived daphnids compared to the corresponding concentration of pure TBZ or expressed based on free TBZ concentration. Similarly, as for PCL, although to a lower extent, the modified release of TBZ from PHB nanoparticles decreased the toxicity on survival. Grillo *et al.* (2010)[22] tested atrazine loaded in the copolymer of polyhydroxybutyrate and hydroxyvalerate and compared it to free atrazine. Genotoxicity was reported to be lower for atrazine in the copolymer nanoformulation due to sustained release.[22]

3.4.4. Commercial formulation. Plot D in Fig. 3 shows the effects on survival of *D. magna* for the commercial formulation (FOLICUR®) compared with pure TBZ. The survival decreased with increased TBZ concentration within the tested concentration range. In lower concentrations of TBZ, the survival (%) of daphnids between FOLICUR® and pure TBZ was similar. With increasing TBZ concentration, the difference between FOLICUR® and pure TBZ became more remarkable. FOLICUR®, as a commercial formulation of TBZ, showed lower toxicity on survival of *D. magna* compared to pure TBZ at higher concentrations of TBZ. In general, the lower toxicity of FOLICUR® can be explained by the lower bioavailability of TBZ in the FOLICUR® formulation in the exposure medium. Commercial formulations of pesticides are mixtures of compounds: a.s., adjuvants, synergists, antagonists, co-formulants, safeners, and solvents. Adjuvants are substances added to pesticide products or pesticide spray mixtures to enhance their performance and/or the physical properties of the spray mixture.[80] The adjuvant used in our commercial formulations might reduce the bioavailability of TBZ, leading to lower toxicity than pure TBZ. Meanwhile the presence of adjuvants and other components in commercial formulations can contribute to toxicity.[80–82] In our case, the commercial formulation's toxicity was lower than pure TBZ. Based on the FOLICUR® safety data sheet (not provided here), it seems that sunflower oil has a high percentage in the composition of FOLICUR® (not mentioned the exact concentration). The data on the effect of sunflower oil on the survival of *D. magna* are not available. Hybská *et al.* (2018)[83] tested vegetable oil for an acute toxicity test on *D. magna* and reported that the EC_{50} was >1 g L^{-1}. Compared with our study, it can be assumed that the concentration of sunflower oil in tested concentrations of FOLICUR® might not be higher than 1 g L^{-1}.

In a toxicity comparison between a nanoformulation of atrazine and its conventional formulation, the nanoformulation was 3.5 times more toxic than the conventional formulation.[43] In the current study, *D. magna* showed less sensitivity to the commercial formulation than to NLC–TBZ and pure TBZ (Table 2).

3.5. The effect of physical properties of nanoformulations and nanocarriers on survival of *D. magna*

It has been reported that smaller nanoparticles usually have a higher toxicity effect on *D. magna* than the same type of nanoparticle in bigger sizes. Nanoparticles with smaller sizes can easily cross cell membranes compared to larger particles.[35,76,84]

Moreover, it has been studied that *D. magna* releases proteins, resulting in eco-corona creation around polystyrene nanoparticles, increasing the nanoparticle's uptake and toxicity. Protein corona (due to nanoparticles bonding to macromolecules in the environment) crucially influenced the nanoparticles' biological behavior/identity.[85] This *D. magna* response influences the feeding rate.[32,86] This phenomenon can happen to all our tested compounds with *D. magna.*

Among our tested NCs, PCL NCs (unloaded with TBZ) were the only ones that showed a concentration (dilution) dependent effects on *D. magna* survival. Their slightly smaller size compared to NLC and PHB NCs, and their chemical composition might contribute to this different effect.

3.5.1. PCL. Plots A and B in Fig. 4 relate the particle concentration and HDD, respectively, of PCL–TBZ and PCL unloaded with TBZ to the survival of *D. magna*. PCL NCs in the tested concentrations prepared using ADaM medium showed toxicity to *D. magna*. The survival (%) increased with decreasing particle concentration and HDD value. As seen in section 3.3, an increase in the dilution of PCL–TBZ and PCL nanoformulation meant a decrease in particle size and HDD, so it could be stated that the survival (%) increased with increasing dilution of both PCL–TBZ and PCL unloaded with TBZ. However, it should be noted that there is a significant difference in the most diluted treatment between NCs in PCL NF and PCL unloaded with TBZ. The higher toxicity in the most diluted treatment in PCL unloaded with TBZ compared with PCL–TBZ can be explained by the higher number of particles, possibly leading to a reduction in O_2 in exposure medium over 48 h.

3.5.2. NLC. Plots C and D in Fig. 4 relate the particle concentration and HDD, respectively, of NLC–TBZ and NLC unloaded with TBZ to the survival of *D. magna*. NLC showed no significant effects on *D. magna* that would depend on the tested concentrations (dilutions). On the other hand, NLC–TBZ showed clear differences in the effect on *D. magna* at different values of particle concentration and HDD that are explainable by differences in the total TBZ concentrations and do not show any relationship to particle concentration or size. Because there are no systematic effects of NLC, other compounds (*e.g.*, Myritol) do not bring remarkable toxicity to *D. magna*. However, in the case of NLC unloaded with TBZ, the effect of other compounds can be the reason for the toxicity to *D. magna*, as there is no TBZ in their composition.

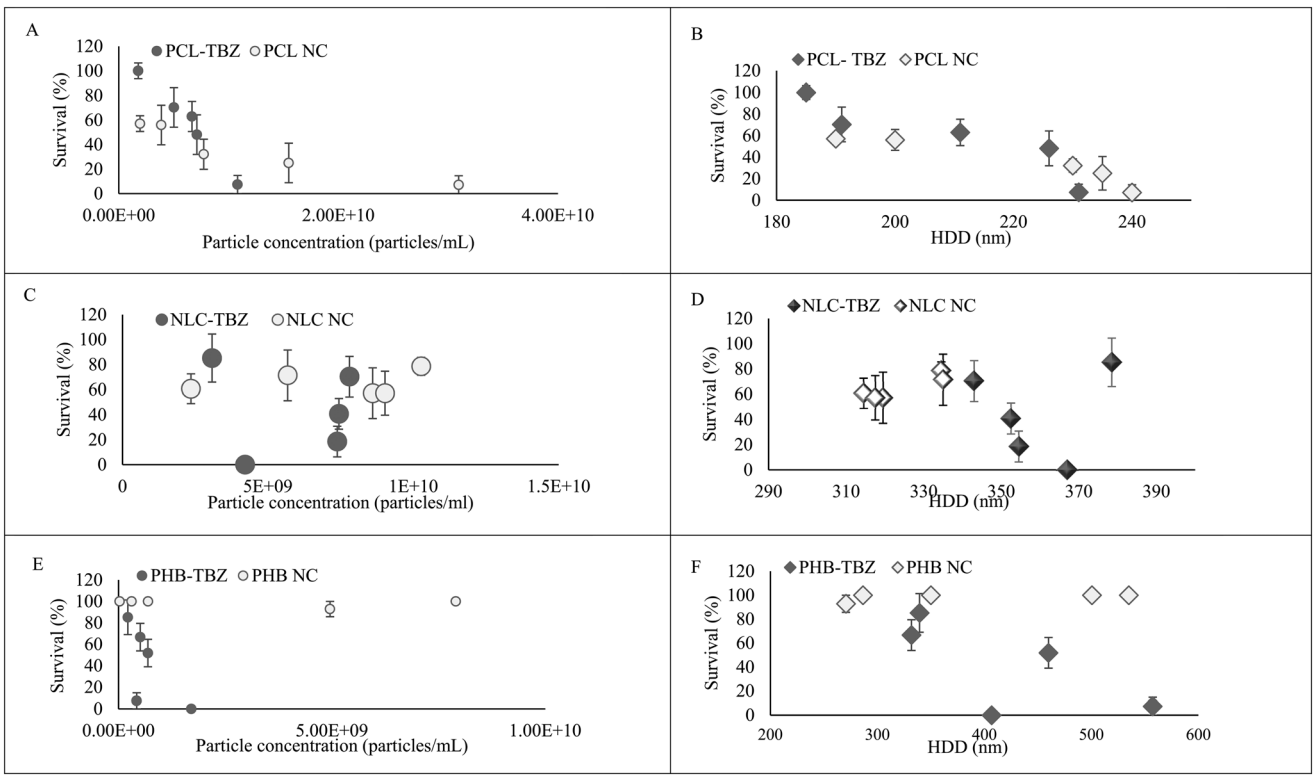

Fig. 4 Survival of *Daphnia magna* in relation to the physical properties of nanoformulations (NFs) loaded with tebuconazole (TBZ) and the corresponding nanocarriers (NCs) unloaded with TBZ in the exposure medium over 48 h. The plots in the same column show the same parameter: particle concentration – plots A, C, E, hydrodynamic diameter (HDD) – plots B, D, F. The plots in the same row show the results for the same carrier: poly-ε-caprolactone (PCL) – plots A and B, nanostructured lipid carriers (NLC) – plots C and D, poly(3-hydroxybutyrate) (PHB) – plots E and F. The values show the mean ± SD (n = 3).

In a study by Albuquerque *et al.* (2021),[80] the NLC unloaded with atrazine in the tested concentrations (0.002, 0.47, 0.97, 1.90 mg L^{-1}) showed toxicity to *Chironomus* larvae, and they suggested that the toxicity could be primarily affected by the composition of the nanocarriers as a possible toxic effect on the larvae.

3.5.3. PHB. Plots E and F in Fig. 4 relate the particle concentration and HDD, respectively, of PHB–TBZ and PHB unloaded with TBZ to the survival of *D. magna*. PHB did not show any significant effects on the survival of *D. magna*, which would depend on the tested concentrations (dilutions). On the other hand, PHB–TBZ showed clear effects that are explainable by total TBZ concentration and do not show any relationship to particle concentration or HDD. The lower toxicity of PHB compared to PCL and NLCs can be explained by their different compositions (explained above). Similar to the present study, size-dependent toxicity might not be observed in other non-target species, as Meredith *et al.* (2016)[61] reported in a comparison test with capsules in different sizes (250 nm, 2200 nm), and free pesticide (λ-cyhalothrin) for 24 h on embryonic zebrafish *Danio rerio*. Capsule size did not show a toxic response to λ-cyhalothrin, but the free form of the pesticide was toxic.

4. Conclusion

In this study, acute immobilization tests with *Daphnia magna* were carried out to address the lethal toxicity of TBZ in different (nano)formulations. Specifically, we assessed the toxicity of a dilution series of three nanopesticides – nanoformulations (NFs) consisting of tebuconazole (TBZ) loaded on poly-ε-caprolactone (PCL), nanostructured lipids (NLC), and poly(3-hydroxybutyrate) (PHB) nanocarriers. Also, all these NCs without loaded TBZ were tested in the same dilutions as the loaded NFs. A commercial formulation of TBZ (FOLICUR®) and analytical grade TBZ were also tested to compare nanopesticide toxicity to conventional plant protection products and pure TBZ.

Our evaluation encompassed not only the TBZ chemistry, considering both total and free concentrations, but also delved into the physical characteristics of the particles, such as their hydrodynamic diameter (HDD) and density.

D. magna responded in a concentration (dilution) dependent manner for all formulations. Increasing total and free concentrations of TBZ caused increasing mortality, and the derived EC_{50} values indicated the toxicity decreasing in the following order: pure TBZ > NLC–TBZ > FOLICUR® > PHB–TBZ ≈ PCL–TBZ.

Notably, the dilution influenced the bioavailability of TBZ in NFs in the exposure medium – in higher dilutions, the bioavailability of TBZ (shown by the difference between total and free TBZ concentration) increased. It can be understood that the decrease in the concentration of stabilization agent used in the composition of NFs likely led to an acceleration in the release of TBZ from NCs.

Comparing the toxicity of NCs unloaded with TBZ might elucidate the mechanisms of toxicity. However, only PCL showed concentration–response effects, while the NLC and PHB NCs were not toxic to *D. magna*. Thus, the PCL–TBZ toxicity might be a combination of NC toxicity and TBZ toxicity. For the other two NFs tested, it seems that TBZ is the primary driver of toxicity.

The physics of particles analyzed in the dilution series revealed a slight dependency of HDD and polydispersity on the dilution. Particle concentration reflected the dilution only in the case of PCL, while for PHB and NLC, the samples faced methodological issues on MADLS.

The accurate mechanisms of toxicity of NFs for our model NFs on *D. magna* survival suffer from scarce data. We assume that the combined toxicity chemically and physically stems from the bioavailability of the a.s., particles' composition, the presence of other compounds in the formulation, changes in particle size, and particle concentration.

Overall, the present study provides valuable insights into the potential ecological impact of different TBZ formulations, particularly in nanoformulations, which can aid in developing safer and more sustainable novel crop protection products. These present results stem from our tested exposure scenario on a lab scale, which might differ with different exposure conditions. Furthermore, the toxicity of formulations (TBZ, FOLICUR®, and NFs) was compared at the same concentration, not considering that nanopesticides may be more efficient and thus used at lower concentrations. Therefore, repeated experiments are highly recommended, considering the real exposure and concentration conditions. Moreover, careful evaluation of the tested formulations and empirical studies like the present one on other non-target organisms in different environment compartments like soil for short and long-term effects will be further necessary. Consequently, a detailed evaluation of nanopesticides' reactivity and toxicity for the safe and sustainable development of nanopesticides in agriculture, along with standardized testing guidelines and robust regulations for their risk evaluation, is crucial.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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