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Statements

Novel formulations for pesticides based on nano-sized carriers loaded with active substances (i.e. nanopesticides) have been developed in recent years to enhance the pesticides' technical, environmental and toxicological properties. In our study, we investigated the behaviour of two tebuconazole nanoformulations in different aquatic media relevant to ecotoxicological testing. This research contributes to a deeper understanding of how the behaviour (release and stability) of nanopesticides is influenced by different factors like concentration of active substance, type of nanocarrier, and conditions in the ecotoxicological test media. Recognizing how these nanoformulations behave is imperative to ensure accurate ecotoxicological evaluations, as changes in active substance behaviour may result in altered bioavailability and ecotoxity results. We believe that our study contributes to building the pathway to reliable assessment approaches for nanopesticides.

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Release and stability of two tebuconazole nanoformulations in different aquatic media

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Abstract

Novel formulations for pesticides based on nano-sized carriers loaded with active substances (i.e., nanopesticides) have been developed in recent years to enhance the technical, environmental, and toxicological properties of pesticides. However, more is needed to know about the behavior of nanopesticides in media used for ecotoxicological tests. In this work, the release and stability of two tebuconazole (TBZ) nanoformulations (TBZ loaded on poly-ε-caprolactone [PCL] or on nano-lipids [NLC]) were evaluated in Milli-Q water (control) and two media used broadly in ecotoxicological tests: reconstituted water (RCW) and Elendt M7 solution. The results suggested that the release of the TBZ depended mainly on the TBZ concentration in the system with faster release at lower concentration levels. TBZ release was also influenced by the type of nanocarrier. For instance, for RCW and concentration of 50 mg/mL, TBZ release from NLC (plateau % = 44.7 ± 2.6) was higher than from PCL (plateau % = 17.8 ± 2.6). Similar trends were also found for other concentrations and the other medium, M7. The colloidal stability of the nanoformulations declined as the concentration of the stabilizing agents decreased. Our results show that it is essential to consider the factors that could influence both the release and stability of the nanopesticides in ecotoxicological media since these phenomena can affect the exposure of organisms to pesticides and, therefore, the results of ecotoxicological tests.

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1. Introduction

The nanoformulation of pesticides (i.e. nanopesticides) may provide several improvements in the properties of the pesticides compared to their conventional formulation such as an increased solubility of the poorly water-soluble active substance, improved dispersion and wettability, protection against premature degradation, and smart release of the active substance.[1–5] These improvements in the properties of the pesticides may lead to an increase in their exposure and effectiveness against pests. Furthermore, since the application would be more precise and specific, a lower dose of nanopesticides would be needed, which would mean a decrease in residues.[5,6] However, the use of nanoformulations of pesticides could have some associated drawbacks. A greater persistence of the active substance in the environment is expected due to the protection against photo- and biodegradation provided by the association with nanocarriers. Higher solubility and greater dispersion in the environment can lead to higher interaction between the nanopesticide and non-targeted organisms.[6] For this reason, it would be fundamental to develop safe-by-design protocols for the preparation and use of nanopesticides before their large-scale applications.[6] Furthermore, the use of pesticide nanoformulations would require a robust regulatory framework to identify the potential risks related to the use of these products. Within the European pesticide regulation, only active substances approved at the European level and their formulations authorized in the individual member states are allowed to be used. However, concerning the environmental fate, the nano-formulations highlight the gap in the current EU legislation, which needs to consider pesticide formulation when the environmental behavior is evaluated.[7]

In the regulatory assessment of ecotoxicity [8,9], both active substances and formulations must be tested using the guidelines of the Organization for Economic Co-operation and Development (OECD) or other standard protocols. However, these guidelines that work for conventional pesticides might not be suitable for pesticide nanoformulations.[10] This is because these guidelines do not consider the novel properties of the nanopesticides resulting from their association with nanometric-sized particles and consequent changes in their behavior during the methods.

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Therefore, the application of standardized procedures assessing nanopesticides would require certain checks and modifications of these protocols that consider these new features. For animal and human health, guidance was provided recently by EFSA to involve the nanospecific aspects in the assessment.[11] Similar guidance would be required for the ecotoxicity assessment of nanoformulated pesticides. The inspiration might be found in the specific OECD recommendations for the aquatic and sediment toxicological testing of nanomaterials.[12] Wang *et al.*[13] stressed it is essential to build a robust framework that can pre-screen, classify and group nanopesticides with similar hazard properties for adequate risk characterization and assessment.[13] Given the novelty of this subject, numerous countries are still discussing on the optimal approach to regulate nanopesticides.[1] For instance, the Australian Pesticides and Veterinary Medicines Authority adopted the principles defined in Kookana *et al.*[14], who assessed whether the presence of the nanoformulation introduces potential differences relative to the conventional active ingredients. The authors also discussed the adaptations to existing environmental risk assessment for use with nanopesticides. They proposed changes in the test methodology and recommended the development of a regulatory framework for nanopesticides.

Previous studies on the risk assessment of nanopesticides showed that the durability, that is a measure of how long a nanocarrier-pesticide complex retained its integrity after applying in the field or in water, is essential to assess exposure patterns.[14-16] Currently, there is no standard protocol to determine the durability of nanopesticides, which is usually determined from the release experiments of active substance from the nanocarriers.[17] The dialysis method is the most applied to nanopesticides. However, in some situations, it does not provide a true measure of the release of the active substance from nanoparticles.[18–20] The "sample and separation" method is an alternative to dialysis that involving dispersing the nanopesticide under sink conditions.[21] This means using a released medium volume three times greater than that required to obtain a saturated solution of active substance and thereby ensuring that the active substance does not precipitate, Then, periodically, samples of the dispersed nanopesticides in the medium are taken and the released active substance (non-associated with nanocarriers) is determined.[22,23]

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Another essential process when conducting ecotoxicity tests is the physicochemical stability of the nanoformulation suspension; that is, the nanoparticles in suspension do not aggregate. Aggregation can lead to the sedimentation of the large particles formed, as well as the loss of beneficial features derived from the nanometric size of the particles. An effective way to evaluate the nanosuspension stability is to measure the particle size and particle-size distribution.

In general, both the release of active substance from nanocarriers and the stability of nanosuspensions are presented as a part of the initial characterization of nanoformulations. It is crucial to determine these parameters considering the possible effect of salinity, organic matter, and pH, among other factors.[24,25] For example, the media used in ecotoxicology tests contain a variety of salts and nutrients that could affect the behavior and fate of the nanopesticides in these media.[11,25] This was the case of the work developed by Kuchovska *et al.*[26] in which the sublethal toxicity of free propiconazole and its polymeric nanoformulation on the embryo-larval stage of the Pacific oyster was evaluated. In this research, the release of propiconazole and the stability of the nanopesticide in the tested medium (seawater) were evaluated. The results showed that the propiconazole nanoformulation was less toxic at the molecular level than free propiconazole, which was associated with the fact that only 44 % of active substance has been released. Castanha *et al.*[25] investigated the behavior of atrazine polymeric nanoparticles in reconstituted water and documented commendable stability, as the particles effectively retained their colloidal properties over an extended period.[25] Similarly, in the case of polymeric nanoparticles within the ADaM medium (used for cultivating/ecotoxicity tests of *Daphnia magna*), there was a notable preservation of their colloidal properties over a 48-hour period.[27] This consistent maintenance of colloidal stability plays a significant role in ensuring uniform and continuous exposure of aquatic organisms to the nanoparticles leading to more reliable and accurate assessments of their ecotoxicological effects, as it minimizes variations in nanoparticle behavior that could otherwise skew the results.[25]

Poly-ε-caprolactone (PCL) is recognized for its biocompatibility and biodegradability, making it a widely utilized polymer in diverse fields such as medical, pharmaceutical, packaging, and cosmetic

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industries.[5,28] The active substance's modified release system, the high encapsulation efficiency of PCL, and its comparatively lower toxicity than free pesticides have been highlighted as key advantages of this polymeric nanocarrier in several studies.[5,27,29,30] Lipid-based delivery systems, another form of nanocarrier, have already established their commercial presence, predominantly in the pharmaceutical sector.[31] Owing to these advantageous properties, such nanocarriers are increasingly attracting interest for applications in the agricultural sector as well.[5, 29,30] Limited data on the fate and behavior of polymeric and lipid nanocarrier in different media used for ecotoxicity studies, underscores the importance and urgency of addressing this knowledge gap.

The main aim of this work was the evaluation of the behavior of nanoformulations composed of tebuconazole (TBZ) associated with poly-ε-caprolactone (PCL) and nanostructured lipid carrier (NLC) nanoparticles in Milli-Q water and in two types of media widely used in ecotoxicological tests: reconstituted water (RCW) and Elendt M7 solution (M7). The release of TBZ from nanocarriers over time and the stability of the nanoparticle dilutions were systematically assessed across a range of concentrations. Thus, the behavior of the nanopesticide was simulated in the same medium and conditions that are used when its toxicity is assessed for different model organisms. The results will contribute to a more robust interpretation of ecotoxicological tests performed on similar nanoformulations and offer suggestions to improve future test design.

2. Materials and methods

2.1. Materials

The analytical standard of TBZ with a purity of > 98.0% was acquired from Sigma Aldrich. Poly-ε-caprolactone (average molecular weight: 80,000), Tween 80, Span 60, glycerol tripalmitate (purity > 85%), and polyvinyl alcohol (PVA) (87-90% hydrolyzed, average molecular weight: 30,000-70,000) were also purchased from Sigma Aldrich (CZ).

2.2. Preparations of nanoformulations.

PCL-TBZ nanoformulation was prepared according to the procedure described by Grillo *et al.*[29] Briefly, 100 mg of PCL, 200 mg of caprylic/capric triglycerides (as Myritol 318), 40 mg of sorbitan monostearate and 10 mg of TBZ were dissolved in 30 mL of acetone. The resulting organic phase

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was slowly poured into 30 mL of an aqueous phase composed of 60 mg of polysorbate 80 surfactant (Tween 80) with magnetic stirring. After 10 minutes of additional stirring, the solvents were evaporated under reduced pressure using a rotatory evaporator to a volume of approximately 20 mL. In the case of NLC-TBZ, the procedure followed was the emulsification method with solvent evaporation, as detailed by De Oliveira *et al.* [32] with some modifications. An organic phase composed of 5 mL of chloroform, 250 mg of glycerol tripalmitate, 75 mg of caprylic/capric (as Myritol 318 form), and 10 mg of TBZ was slowly added into 30 mL of 1.25% PVA aqueous solutions. The pre-emulsion was dispersed with a high-speed Ultraturrax homogenizer at 14000 rpm for 7 min. Then, the solvent was evaporated in the rotatory evaporator to a volume of approximately 20 mL.

2.3. Characterization

2.3.1. The total concentration of TBZ

The total concentration of TBZ was determined according to method described in Grillo *et al.*[29] by extracting 200 µL of each nanoformulation with 19.8 mL of acetonitrile (in triplicate). The mixtures were shaken for 30 seconds in a Vortex mixer, and the extracts were dissolved 10 times with acetonitrile, filtered through nylon filters (0.22 µm), and analyzed by HPLC-MS/MS (HPLC-MS/MS operating conditions are provided in the Supplementary information).

2.3.2. Encapsulation efficiency

The encapsulation efficiency (EE) is the amount of pesticide associated with the nanocarriers in relation to the total amount of pesticide contained in the nanoformulation. EE was calculated from the following expression:

$$EE\ (\%) = \frac{m_{associated}}{m_{total}} = \frac{m_{total} - m_{free}}{m_{total}} \qquad \text{Eq.1}$$

Where m_{total} is the mass determined from the total concentration (section 2.3.1.), and $m_{associated}$ is the mass of TBZ associated with the nanocarriers, which is calculated as the difference between m_{total} and the mass of free pesticide not loaded into the nanocarriers (m_{free}). The free mass of TBZ in the nanoformulations was obtained by centrifugal ultrafiltration. For this, 0.5 mL of each nanoformulation was added to centrifugal ultrafiltration devices containing a 30 kD regenerated

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cellulose filter (Microcon®, Millipore), and the devices were centrifuged at 14000 x g for 30 min. The filtrates were diluted 100-fold and analyzed by HPLC-MS/MS.

2.3.3. Particle size, polydispersity index, and zeta potential

Hydrodynamic diameter (D_H), polydispersity index (PI), and ζ-potential of the nanoformulations were determined by dynamic light scattering (DLS) using a Malvern® Zetasizer Nano-ZS with detector at a fixed angle of 173°. Before the measurements, the nanoformulation was diluted (1:100 and 1:1000 for PCL-TBZ and NLC-TBZ, respectively) with Milli-Q water (refractive index = 13.30 and viscosity = 0.8872). All the measurements were performed in triplicate.

2.3.4. Morphology of the nanoparticles (Transmission Electron Microscopy)

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

2.4. Release studies of nanoformulations at different concentrations of active substance in different media

The release of TBZ from the nanoparticles was evaluated by the "sample and separation" method at different total concentrations of TBZ. The media used were Milli-Q water, reconstituted water (RCW), and Elendt M7 solution (M7), the latter two prepared according to OECD guidelines.[33,34] The compositions of RCW and M7 media are presented in tables S1 and S2 in the Supplementary information. The pH values of RCW and M7 were 7.7 and 7.8, respectively. Both RCW and M7 are media commonly used in ecotoxicology tests with model organisms such as fish,[35,36] *Daphnia magna*[33,34] and larvae of *Chironomidae,*[37] among others. Release experiments were carried out at three total concentrations of TBZ: 50 µg/mL, 5 µg/mL, and 0.5 µg/mL. These values were chosen to cover concentrations both above and below the solubility of TBZ in water (36 µg/mL), as well as concentrations close to those used in some relevant sub-lethal ecotoxicological tests of this fungicide on model organisms such as *Daphnia magna,*[38] *Danio rerio*[39] and *Gammarus fossarum.*[40]

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These concentrations were obtained by diluting the nanoformulation stock suspensions with autoclaved media using sterile tips. Sterilized media blanks without nanoformulation were also prepared. After preparing every dilution, the bottles were closed, shaken, and immediately, the first sample was taken (t = 0). Then, the suspensions were shaken on an orbital shaker protected from light at 100 rpm, and at selected times (4, 8, 24, 48, 240, and 672 h), the bottles were removed from the shaker and sampled. The sampling points were selected considering the duration of some of the most used ecotoxicological tests, such as acute (24 and 48 hours) and reproduction (21 days) tests with *Daphnia magna*.[33,34] Both the dilution of nanoformulations and the sampling were carried out in a laminar flow box to maintain sterile conditions and thus avoid the possible biodegradation of the pesticide and/or the nanocarriers. At each sampling point, 0.5 mL of nanoformulation dilutions and blank were poured into Microcon® device (in triplicate) and centrifuged at 14,000 x g for 30 min. Then, the filtrates were removed, diluted with acetonitrile, and stored in the freezer until measured by HPLC-MS/MS. Along with the free mass of TBZ, the total concentration of TBZ was also determined at each sampling point. For that, in triplicate, a sample of each nanoformulation dilution was extracted with acetonitrile, as described in section 2.3.1.

From the total amount of TBZ (m_{total}) and free amount of TBZ (m_{free}) in the dilutions of the nanoformulations, the cumulative release of TBZ was determined at each sampling point according to the expression:

$$Cumulative\ release\ (\%) = \frac{m_{free}}{m_{total}} \times 100 \qquad \text{Eq.2}$$

2.5. Stability study of nanoformulations in different media at different concentrations

The stability of nanoformulations was evaluated by determining changes in the hydrodynamic diameter (D_H) and polydispersity index (PI) of the diluted nanoformulations over time in the different media. The increase of these two parameters indicates the aggregation of the nanoparticles and, consequently, the destabilization of the suspensions. The procedures described above (section 2.4) for preparing the diluted nanoformulations and their subsequent sampling were repeated to assess the D_h and PI values over time. The initial sampling point was

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immediately after dilution (t = 0), followed by subsequent sampling at 24, 240, and 672 hours from the dilution of the nanoformulations. Samples were measured in triplicate (three measurements of each sample) by DLS as described above in section 2.3.3, but without diluting the samples.

2.6. Statistical analysis

Statistical analysis was carried out using Excel and SigmaPlot 14. All the results were expressed as mean ± standard deviation to indicate inter-replicate variability. The standard deviations of the percentage of pesticide released were calculated using the propagation of error rules.

3. Results and discussion

3.1. Characterization of nanoformulations

The characterization of the nanoformulations was carried out immediately before the experiments. Table 1 contains the physicochemical characteristics of each nanoformulation, including the values of the total concentration of TBZ, encapsulation efficiency (EE), hydrodynamic diameter (D_H), polydispersity index (PI), ζ potential, and pH.

The EE values of both nanoformulations were very high (96.9 ± 2.3% and 92.0 ± 5.0% for PCL-TBZ and NLC-TBZ, respectively) indicating a great affinity between TBZ and the nanocarriers, probably due to the high hydrophobicity of both the fungicide and the chemical components of the nanoparticles (PLC polymer, caprylic/capric triglycerides and glyceryl tripalmitate).

The D_H of PCL-TBZ (241.2 ± 0.8 nm) and NLC-TBZ (343.5 ± 5.6 nm) are within the previously reported particle size range for each type of nanoparticle: 250 – 500 nm for PCL particles[41] and 10 – 1000 nm for NLC particles.[42]

PI values indicate the monodispersity of the D_H distributions in a colloidal suspension. Values of PI ≤ 0.2 in suspension of polymeric nanoparticles and PI ≤ 0.3 for suspensions of lipid-based nanocarriers imply a narrow size distribution, that is, a monodisperse distribution.[43] This is the case of our nanoformulations, where the PI values were 0.24 ± 0.01 and 0.23 ± 0.01 for PCL-TBZ and NLC-TBZ, respectively.

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ζ potential values greater than ±30 mV entail repulsive forces between particles that are strong enough to prevent their aggregation.[44] In the case of our PCL and NLC nanoparticles, the ζ potentials were -37.5 ± 0.5 mV and -37.9 ± 0.4 mV, respectively. Thus, these values mean that the nanoformulations are electrostatically stabilized. It is worth to highlight that the presence of stabilizing agents (surfactants and polymers) in the suspensions would also contribute to stabilizing our nanoformulations by steric and depletion effects. In the PCL nanoparticles, the negative values of ζ potential can be attributed to terminal carboxylic groups from the polyester polymer in the presence of a non-ionic stabilizing agent (Tween 80).[29,41]

Based on the results obtained in the characterization, it is concluded that both nanoformulations were stable before the experiments, with D_H values within the nano-size range, monodisperse particle size distribution, and ζ potential values that indicate electrostatic stabilization.

Transmission electron microscopy (TEM) micrographs, illustrated in Fig S3 (in the Supplementary materials), show the morphological features of the PCL and NLC nanoparticles. The focus of this analysis is to clarify the uniformity of the particles and the absence of aggregation. A notable alignment between TEM analysis and DLS results is observed, validating the reliability of our findings. Furthermore, utilizing both TEM and DLS aids in gaining a thorough insight into the particle system.

3.2 Release studies of PCL-TBZ and NLC-TBZ at different concentrations of the active substance in different media.

3.2.1. Influence of the medium on the release of TBZ

The pH values of diluted PCL-TBZ range from 7.0 to 7.8 and 6.9 to 7.4 in RCW and M7, respectively. In the case of NCL-TBZ dilutions, the pH values were 6.9-7.4 for both media. The release profiles of TBZ from PCL nanoparticles at a given concentration were similar in the different media, reaching a similar release plateau percentage (Fig 1a). After diluting the stock suspension of PCL-TBZ nanoformulation to obtain the desired concentrations, the plateau percentage of TBZ was reached in less than one hour. The rest of TBZ must remain associated with the nanocarriers until, at least, the end of the experiment since no decrease in total TBZ concentration was detected in

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the diluted nanoformulations over time (Fig. S1 and S2). From the principal release mechanisms of active substances from nanocarriers (erosion-, solvent-, and diffusion-controlled release),[45-47] the primary expected mechanism must be the one based on diffusion as a result of the concentration gradient between the nanoparticles and the release medium. Erosion- controlled release can be due to biotic (enzymatic) and/or abiotic (hydrolysis, photolysis…) degradation.[4] Our release experiments were performed in sterile conditions to avoid the possible biodegradation of the polymeric and lipid nanocarriers. However, hydrolysis-controlled release may occur in the time frame of the experiments (672 hours). Solvent-controlled release of TBZ is not expected. This mechanism consists of the transport of the solvent within the nanocarrier, followed by the dissolution of the active ingredient and its release through a process of swelling and relaxation of the nanocarrier matrix. Due to the hydrophobicity of the polymeric and lipid nanocarriers, the entry of water inside the nanoparticles would not be favored and, therefore, this mechanism would not occur.

For NLC-TBZ, no difference in the release profile was observed at a given concentration in the different media except for 50 µg/mL dilution, whose plateau percentage in Milli-Q Water (36.9 ± 4.4% at 672 hours) was lower than of RCW (44.7 ± 2.7% at 672 hours) and M7 (47.4 ± 1.6% at 672 hours).

These results suggest that the release of TBZ from the tested nanocarriers was not influenced by the chemical composition of the media. In other words, the media did not affect the durability of the complexes TBZ-nanocarrier.

3.2.2. Effect of the dilution factor on the release of TBZ

The release of TBZ from both nanocarriers was strongly influenced by the final concentration of the active substance after dilutions of stock nanoformulations. Thus, the percentage of TBZ released was greater as dilution increased (i.e., as the total concentration of TBZ decreased). This suggests a major contribution of diffusion as the release mechanism of the fungicide since diffusion is driven by differences in concentration between the medium and the surface of the particles. Therefore, higher dilution means a higher concentration gradient and, hence, greater

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and faster diffusion[4]. Furthermore, another possible reason for the significantly lower release percentage of 50 µg/mL dilution compared to the other dilutions would be that this concentration exceeded TBZ solubility in water (36 µg/mL). Surfactant from the nanoformulation suspension is expected to increase the solubility of TBZ, but the effect may not be sufficient to increase the release process significantly. Conversely, the release could be enhanced at concentrations of 5 µg/mL and 0.5 µg/mL as they are below the water solubility of the TBZ.

3.2.3. Impact of nanocarrier type on the release of TBZ

For a given concentration and medium, the percentage of TBZ release values at the plateau was higher from NLC than from PCL nanoparticles (Fig. 1a and b). Considering that both nanoformulations contain the same liquid lipid (i.e., Myritol), the differences in the release must be related to the affinity of the TBZ for the polymer PCL and the solid lipid glyceryl tripalmitate. Due to the hydrophobicity of the pesticide, the interaction between TBZ and the solid lipid is expected to be higher than with the polymer. Ventury *et al.*[48] studied the release of imiquimod from PCL nanocapsules with a copaiba oil core and from NLC nanoparticles consisting of cupuaçu seed butter as a solid lipid and copaiba oil as a liquid lipid. The release of imiquimod from PCL nanocarrier was faster than that from NLC and was attributed to a high drug affinity for the solid lipid. In our study, however, we observed a contrasting behavior: there was a higher release of TBZ from NLC compared to the PCL nanocarrier.

We obtained the same result in our previous work in which the release of TBZ from several nanocarriers, including PCL and NLC, was assessed in Milli-Q water by dialysis method.[49] The faster release observed from NLC than from PCL nanocarriers was attributed to an imperfectly solid matrix structure of the nanoparticles originating as a consequence of the preparation procedure.

The results show that the release of TBZ from the nanoformulation did not depend on the characteristics of the medium but on the total concentration of the active substance after dilution and the type of nanocarrier.

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3.3. *Stability of the nanoformulation suspensions over time in different media*

The stability of the diluted nanoformulations was monitored by measuring changes in D_H and PI values over time as indicators for the formation of aggregates. The destabilization of colloidal suspension results from attractive van der Waals interactions between particles.[50] Thus, to achieve the stability of these suspensions, it is necessary to have effective repulsion mechanisms that overcome these attractive forces. There are three main mechanisms that lead to colloidal stability: electrostatic, steric, and depletion stabilizations.[51] Electrostatic stabilization is based on repulsion forces caused by the overlapping of electrical double layers that surround the particles. Steric stabilization is achieved by the sorption of polymers or surfactants on the particle surface. This steric effect keeps the particles sufficiently separated to reduce the attractive van der Waals interactions. The last stabilization mechanism to consider is depletion, which is a consequence of the presence of non-adsorbing polymers above a certain critical concentration.

Fluctuations in ζ potential values over time may reflect the loss of electrostatic stabilization. Thus, when ζ potential is greater than - 30 mV or less than + 30 mV (for negatively or positively charged particles, respectively) the particles would not have the necessary charge to repel each other, which would lead to their aggregation.[44,52] In our tests, the ionic strength of the media tested could alter the ζ potential measurements,[44] and the ζ potential was therefore not applicable to assess the stability of the nanoformulations over time.

For PCL-TBZ in Milli-Q water, D_H and PI values remained approximately constant at all concentrations during the entire time frame of the experiments (Fig. 2a and b). Similarly, in the M7 medium, D_H and PI values were similar at all times and concentrations, except for the highest concentration and latest time point (50 µg/mL at 672 hours), where both D_H and PI values were significantly higher. For RCW, the D_H was similar for all the concentrations over time, but the PI increased after 672 hours for all three concentrations (and for 5 µg/mL, also after 240 hours). It was observed that the increase in PI was greater with the greater dilution of the nanoformulation. This increase in PI could indicate the beginning of the aggregate formation that would lead to a polydisperse distribution.

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The stability of NLC-TBZ nanoformulation was lower than that of PCL-TBZ; both D_H and PI varied over time, medium, and concentration (Fig. 3a and b). For NLC-TBZ diluted in Milli-Q water, a significant increase in D_H (from 400 nm to 555 nm) and the PI values (from values around 0.30 at the beginning to values around 0.40-0.45 after 240 to 672 hours) was detected in the less diluted nanoformulation (50 μg/mL) over time. In contrast, in the other media, the effect of the dilution on the stability of the nanoformulation was the opposite; that is, an increase in D_H and PI values occurred over time at the lowest concentration (0.5 μg/mL). The decreased stability of PCL-TBZ in RCW and NLC-TBZ in RCW and M7 with increasing dilution of the nanoformulations could be attributed to the strong dependence of steric and depletion stabilization on the concentration of the stabilizing agent (polymer or surfactant) in the nanoformulations. When the concentration of stabilizing agent is high enough, it saturates the entire surface of the particles, forming a layer of the stabilizing agent surrounding the particle. This layer overlaps with those of neighboring particles as they approach each other, resulting in steric repulsion between them.[53] Once all particle surfaces are saturated with the stabilizing agent, the non-adsorbing molecules can concentrate near the depletion zone. This results in a higher osmotic pressure outside (in the medium) than between the particles, resulting in a weak depletion attraction that gives rise to depletion flocculation.[50] If the concentration of free stabilizing agent increases to exceed depletion flocculation, depletion stabilization would be achieved.[54,55] Therefore, in our experiments, when the concentration of surfactant (Tween 80 and Span 60 for PCL-TBZ) or polymer (PVA for NLC-TBZ) decreased by the dilution of stock nanoformulations, the two phenomena described above could contribute to the destabilization of the diluted nanoformulations. That is, bridging flocculation between surfactant or polymer molecules may occur due to insufficient stabilizing agent concentration to completely cover the entire available surface of the particles,[53,55] and depletion attraction appears because of a reduction in the concentration of non-adsorbing stabilizing agent.[55]

For both PCL-TBZ and NLC-TBZ nanoformulations, the effect of dilution on the stability was media-dependent, with destabilization in media containing ions (RCW and M7). Generally, an

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increase in the concentration of salts causes a destabilization of colloidal systems due to the collapse of the electrical double layer, which reduces the electrostatic stabilization.[56,57]

The destabilization of the nanoformulations as concentration decreased was more noticeable for NLC-TBZ than for PCL-TBZ. For instance, D_H values could not be measured for NCL-TBZ at the lowest concentration and last sampling points due to a high PI that fell outside of the quality requirements of the instrument. This was probably due to the use of PVA as a stabilizing agent in NCL-TBZ since the presence of salts in the medium can reduce the solubility of the polymer (decreased in stabilizing agent concentration), resulting in a reduction in steric and depletion stabilization as explained above.[56,57]

4. Conclusion

In this work, the release and stability of two TBZ nanoformulations (PCL-TBZ and NLC-TBZ) were assessed in Milli-Q water and in two media widely used in ecotoxicological tests. The release of TBZ from PCL and NLC nanoparticles did not depend on the media. However, the release was strongly influenced by the concentration of TBZ: faster release (short durability) was observed at lower concentrations. These results are crucial because the sub-lethal tests of TBZ on model organisms generally require concentrations close to those used in our experiments. Therefore, it would be expected that, in these ecotoxicological tests, the behavior and toxicity of the TBZ nanoformulations would be equivalent to those of pure active substance since all the nanospecific properties of the TBZ-nanocarrier association would be lost quickly after preparation of solutions. Likewise, the stability of the nanoformulations was also dependent on concentration. Generally, as the dilution of the nanoformulation increased, the concentration of stabilizing agents (Tween 80, Span 60 for PCL-TBZ and PVA for NLC-TBZ) decreased, resulting in a reduction of steric and depletion stabilization that led to the aggregate formation. The medium and the polymer type also influenced this destabilization.

These results indicate that the conditions necessary for carrying out the ecotoxicological tests may affect the durability and stability of pesticide nanoformulations. Therefore, it is essential to evaluate the behavior of nanopesticides in the test medium before performing an ecotoxicological

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test and, if necessary, to include modifications in the protocol. Based on the results obtained in our experiments, a previous step could be to verify that the solubility of the stabilizing agents in the medium is sufficiently high. Otherwise, it becomes imperative to find alternative stabilizing agent with greater solubility to avoid potential destabilization of the nanoformulation.

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Table 1. Total concentration of tebuconazole (TBZ), hydrodynamic diameter (D_H), polydispersity index (PI), ζ potential, and encapsulation efficiency (EE) of the nanoformulations of tebuconazole (TBZ) loaded on poly-Ɛ-caprolactone (PCL) and nanostructured lipid nanocarrier (NLC) nanoparticles. The values show the mean of 3 replicates ± standard deviation.

Nanoformulation	**Total concentration (µg/mL)**	**EE (%)**	**D_H (nm)**	**PI**	**ζ potential (mV)**	**pH**
PCL-TBZ	314.0 ± 5.2	96.9 ± 2.3	241.1 ± 0.8	0.24 ± 0.01	-37.5 ± 0.5	7.0
NLC-TBZ	313.5 ± 11.5	92.0 ± 5.0	343.5 ± 5.6	0.23 ± 0.01	-37.9 ± 0.4	5.0

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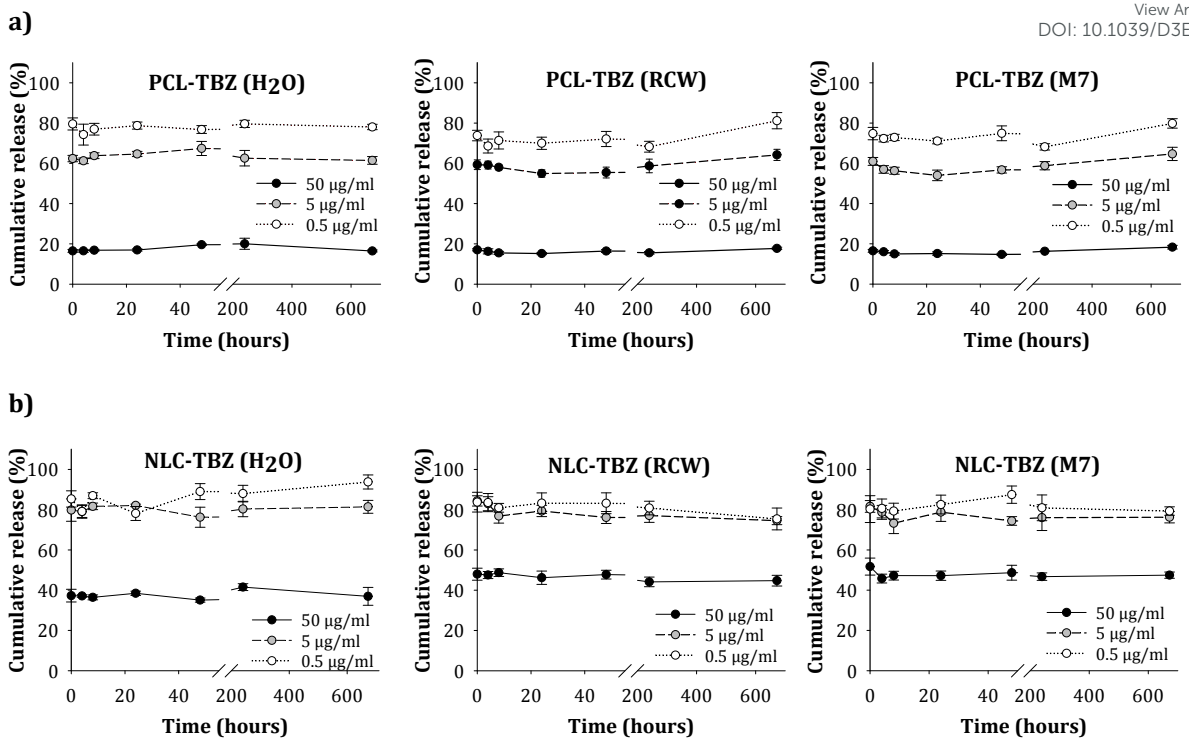


Fig. 1 Cumulative percentage release of tebuconazole (TBZ) from a) poly(Ɛ-caprolactone) (PCL) nanocarrier and b) nanostructured lipid carrier (NLC) at different total TBZ concentrations in the three media: Milli-Q water (H_2O), reconstituted water (RCW) and Elendt M7 solution (M7). Error bars correspond to the standard deviation of triplicate measurements. The lines connect the points and do not represent any model.

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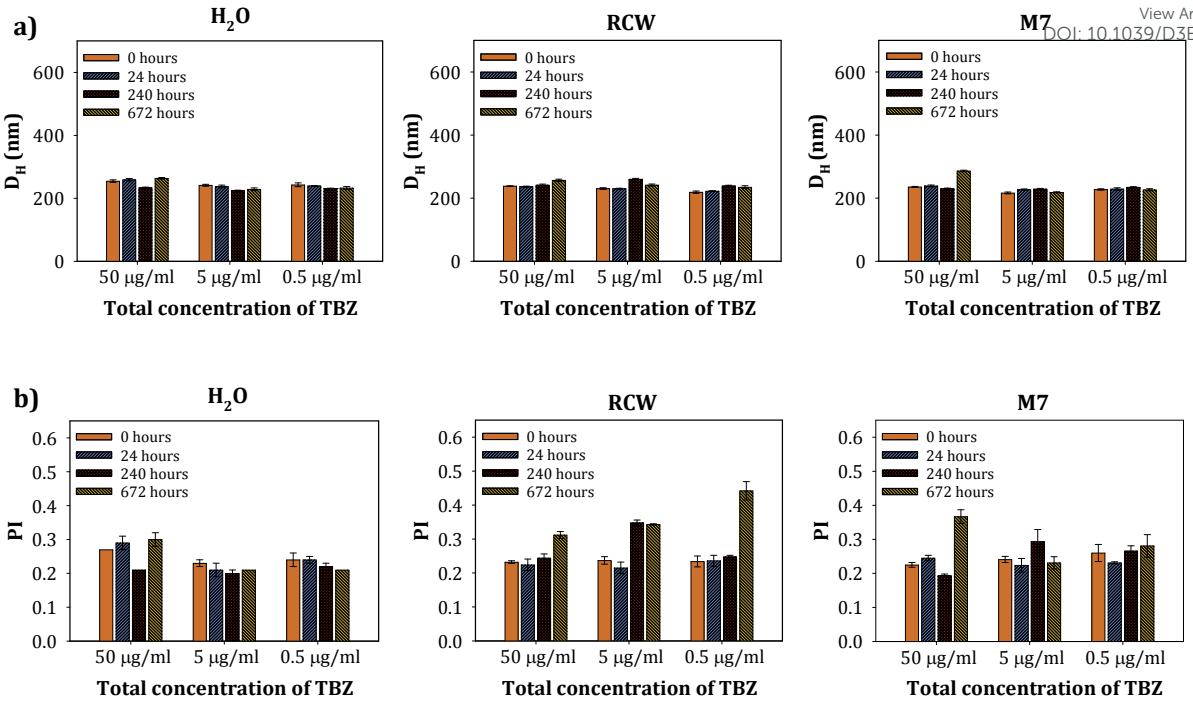


Fig. 2 Stability of **PCL-TBZ** nanoparticles over time (0, 24, 240, and 672 hours) in the three media: Milli-Q water (H_2O), reconstituted water (RCW), and Elendt M7 solution (M7) by a) hydrodynamic diameter (D_H) and b) polydispersity index (PI) measurements (n=3).

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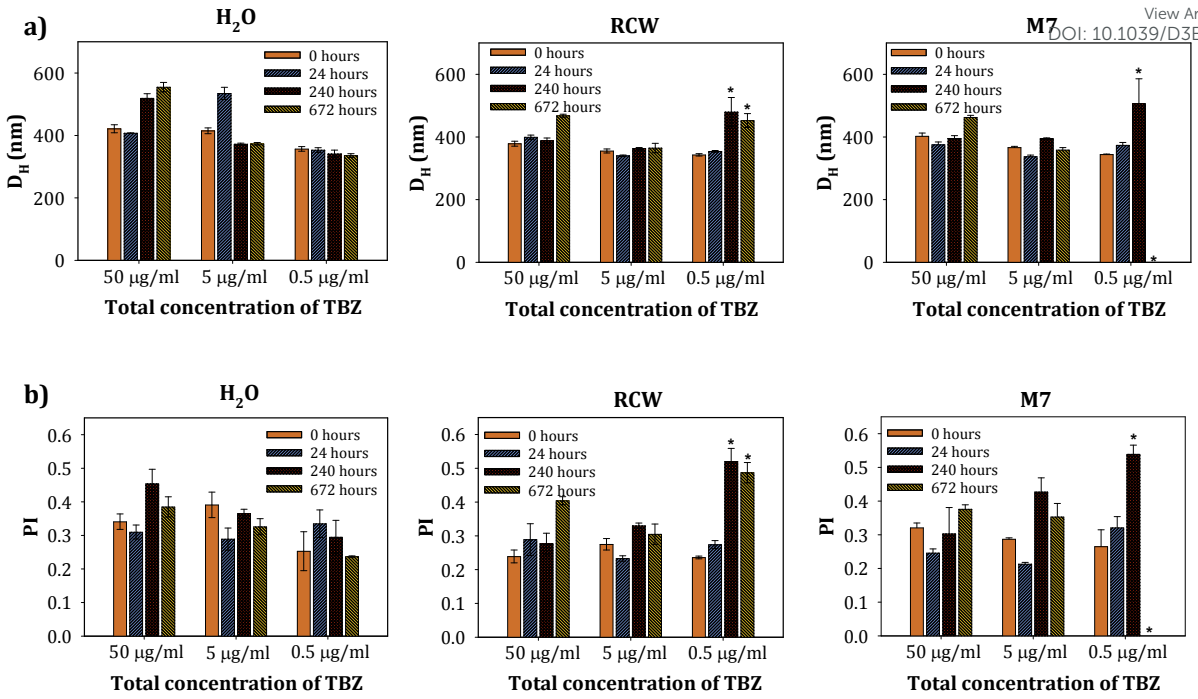


Fig. 3 Stability of **NLC-TBZ** nanoparticles over time (0, 24, 240, and 672 hours) in the three media: Milli-Q water (H_2O), reconstituted water (RCW), and Elendt M7 solution (M7) by a) hydrodynamic diameter (D_H) and b) polydispersity index (PI) measurements (n=3). The symbol * indicates that the result did not meet the quality criteria stipulated by the instrument Malvern® Zetasizer Nano-ZS.

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