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How Can Nanoplastics Affect the Survival, Reproduction, and Behaviour of the Soil Model *Enchytraeus crypticus*?

Angela Barreto †, Joana Santos †, Mónica J. B. Amorim and Vera L. Maria *

Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal; abarreto@ua.pt (A.B.); joanasilvasantos@ua.pt (J.S.); mjamorim@ua.pt (M.J.B.A.)

* Correspondence: vmaria@ua.pt; Tel.: +351-234-370-350; Fax: +351-234-372-587

† Equal first authors—Angela Barreto and Joana Santos.

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Abstract: Nanoplastics (NPls) are ubiquitous in terrestrial environments, with numerous consequences for biodiversity and ecosystems. Research is urgently required to clarify the NPls environmental behaviour, fate and ecotoxicological effects to soil ecosystems. The aim of this research was to assess and comprehend the effects of polystyrene NPls to the terrestrial species *Enchytraeus crypticus* using survival, reproduction and avoidance behaviour as endpoints. A range of concentrations, 0.015 to 1500 mg NPls/kg LUFA 2.2 (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Germany) soil, was tested. Due to the presence of tween 20 and sodium azide (NaN_3) on the NPls dispersion, the effects of these compounds were also assessed separately. After 21 d, 1200 and 1500 mg/kg NPls dispersion had significant effects on the organims survival and/or reproduction. However, these effects may be mainly associated with tween 20 and NaN_3 present in the NPls dispersion and not with NPls themselves. After 48 h, there was a tendency of the organisms to avoid the NPls spiked soils, being this response significant at 0.015 mg/kg although a reduced avoidance behaviour was observed as NPls concentration increased. The present study provides screening data on the effects of NPls, alone and considering the presence of other compounds like the solvents, which is essential for regulators and strategic management of plastic pollution.

Keywords: plastics; terrestrial invertebrates; mortality; reproductive output; avoidance

1. Introduction

In 2016, the production of plastic around the world, mainly used for packaging, exceeded the 320 million tons. This production rise was accompanied by an unprecedented exponential increase in the occurrence of plastic waste in both soil and aquatic environments [1]. Once in the environment, plastics may breakdown through several processes, for instance mechanical forces and photo-oxidative processes, into microplastics (MPls; smaller than 5 mm) and nanoplastics (NPls; sized between 1 to 1000 nm), changing their physicochemical characteristics, and thereby their bioavailability and potential impact on the organisms [2]. MPls and NPls occur in soil at higher levels—at least a factor of four—than in marine environments [3]. However, the presence of MPls and NPls in terrestrial ecosystems and their toxic effects to the biota have received little attention [4]. In fact, plastic debris remaining on the soil surface are more exposed to UV (ultraviolet) light than the ones present in the marine compartments, leading to a high presence of MPls and NPls on the terrestrial ecosystems [5]. For instance, around 4000 MPls particles.kg^{-1} of dry sludge (~0.04%) were found at European agricultural and landfill sites [6]. Although it is known the widespread presence of NPls into the environment, data concerning their environmental levels are still missing due to the limitations of the analytical techniques associated

with the NPls small size [2]. NPls concentrations < 15 µg/L have been predicted to be environmentally relevant concentrations [7]. Presenting a much higher surface area to volume ratio than MPls, NPls may be more easily ingested (depending e.g., on their aggregation state) and may induce greater toxic effects to the organisms [8]. Despite the increasing number of studies regarding the toxicity of NPls to aquatic invertebrates (e.g., *Mytilus galloprovincialis* [2,9] and *Daphnia magna* [10–13]), concerning soil invertebrates only one study was found to the model species *Enchytraeus crypticus* [5]—Table 1. Therefore, it is crucial to increase the knowledge about the toxicity of NPls to terrestrial organisms, contributing to a better environmental risk assessment of NPls to the soil ecosystems.

Table 1. Studies assessing the effects of microplastics and nanoplastics on soil invertebrates.

Characteristics of the Particles	Species	Characteristics of the Exposure	Assessed Endpoints	Main Findings	Rf
Nanoplastics (NPls)					
Polystyrene, 0.05 to 0.1 µm	*E. crypticus*	Via food 0.025, 0.5 and 10% 7 days	Reproduction Growth Gut microbiome NPls detection	NPls reduced the weight, had a hermetic-like effect on reproduction and changed the gut microbiome.	[5]
Microplastics (MPls)					
High-density polyethylene, 1.32 ± 0.72 mm	*L. terrestris*	Via soil spiked 236, 1261 and 4505 mg/kg with 0.35 wt % zinc 28 days	Survival Growth MPls adsorption /desorption capacity and accumulation	MPls had the potential to act as vector for increasing uptake of zinc.	[14]
Polyethylene, 250 to 1000 µm	*E. andrei*	Via soil spiked 62.5, 125, 250, 500 and 1000 mg/kg 28 days	Reproduction Survival Growth Histopathology and molecular analysis	Severe histological damages in the gut. Molecular changes related to immune system.	[15]
Polyethylene, < 150 µm	*L. terrestris*	Via soil spiked 7, 28, 45 and 60% 60 days	Reproduction Survival Growth MPls ingestion	Mortality increased and growth rate was reduced. MPls were concentrated in cast.	[6]
Polyvinyl chloride, 80 and 250 µm	*F. candida*	Via soil spiked 1 g/kg 48 h	Reproduction Growth Gut microbiome Isotopic turnover	Growth and reproduction were inhibited. MPls changed the gut microbiome. Enhanced δ15N and δ13C values.	[16]

Rf–reference.

Due to the urgent need to prioritize research dealing with this topic, the aim of this study was to assess the toxicity of polystyrene NPls to the standard and ecologically relevant soil model *Enchytraeus crypticus*. Toxicity was measured using survival and reproductive outputs as endpoints. Additionally, the avoidance behaviour of *E. crypticus* after the exposure to NPls was also evaluated. Enchytraeids live in the litter layer and the upper mineral soil where they feed with fungal hyphae, microorganisms and dead organic matter (OM) [17]. Moreover, they vigorously contribute to the acceleration of OM decomposition and nutrient recycling processes. Being a soft-bodied soil invertebrate, uptake is made via ingestion (e.g., food and soil particles) as well as via the body surface or dermis (used for gaseous exchange and water uptake) [18]. Many studies already demonstrated *E. crypticus* as a valuable bioindicator of contaminated environments from a wide range of anthropogenic sources (e.g., metals and nanoparticles) [19–21].

2. Material and Methods

2.1. Test Organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta), Westheide & Graefe, 1992, was used for the tests. According to Directive 2010/63/EU of the European Parliament and of the Council of 22/9/2010, invertebrates, like *E. crypticus*, are permitted biological models for scientific experimentation and are free of Ethical Statement. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four salt solutions: 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl and 0.75 mM $NaHCO_3$, at a temperature of 20 ± 1 °C with a 16 h:8 h light:dark photoperiod. Cultures were fed on ground-autoclaved oats twice per week.

2.2. Test Materials and Characterization

Polystyrene nanoplastics (NPls) dispersion (4.9% solids in deionized water with 0.1% tween 20 and 2 mM sodium azide (NaN_3)) visible dyed (crimson red) was acquired from Bangs Laboratories, Inc. According to supplier, NPls had mean diameter of 49 nm and a surface area of 1.361×10^{14} $\mu m^2/g$. NPls, in stock dispersion and diluted in standard ISO (International Organization for Standardization) reconstituted water [22] (to obtain the concentrations used in the tests), were characterized by UV–Vis spectrum (Cintra 303, GBC Scientific), by Fourier transform infrared spectroscopy (FTIR) spectrum (Bruker Tensor 27), by hydrodynamic size assessed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern) and by zeta potential (ZP) assessed by electrophoretic light scattering (Zetasizer Nano ZS, Malvern). The Zetasizer Nano ZS (Malvern) also allowed to obtain the polydispersity index (PDI) of the NPls dispersions.

2.3. Exposure via Soil

2.3.1. Test Soil and Spiking Procedures

The natural standard LUFA 2.2 soil (Speyer, Germany) was used for the tests, presenting the main characteristics: pH (0.01 M $CaCl_2$) = 5.8, organic carbon = 1.71%, cation exchange capacity = 9.2 meq/100 g, maximum water holding capacity (WHC) = 44.8% and grain size distribution of 7.2% clay, 8% silt and 77.5% sand.

The soil was dried (48 h; 60 °C) before use. The control soil was prepared by adding deionized water to adjust to the adequate moisture content (50% of the maximum WHC). For the enchytraeid reproduction test (ERT), soil spiking was performed using the following nominal concentrations: 0.015, 1.5, 15, 150, 300, 450, 600, 900, 1200 and 1500 mg NPls/kg soil (Table 2).

Table 2. Correspondence between the tested nominal concentrations of polystyrene nanoplastics (NPls) and the number of particles.

NPls Nominal Concentration (mg/kg)	Number of Nanospheres/kg
0.015	$0.000036825 \times 10^{16}$
1.5	0.0036825×10^{16}
15	0.036825×10^{16}
150	0.36825×10^{16}
300	0.7365×10^{16}
450	1.10475×10^{16}
600	1.475×10^{16}
900	2.2095×10^{16}
1200	2.946×10^{16}
1500	3.6825×10^{16}

Due to the presence of tween 20 (0.1%) and NaN_3 (2 mM) on the NPls dispersion, the effects of the combination of these two compounds were also assessed, adding the same volume of the dispersant

aqueous solution (0.1% tween 20 and 2 mM NaN_3) as those used in the concentrations of NPls. The tested concentrations of tween 20 and NaN_3 are presented on Table 3. Based on the results from ERT, for the avoidance test, soil spiking was performed using the following nominal concentrations: 0.015, 1.5, 150, 300, 450 and 600 mg NPls/kg soil and a dispersant control (tween 20 + NaN_3), adding the same volume of the dispersant aqueous solution as used with the highest concentration of NPls (600 mg/kg). The NPls dispersions or dispersant aqueous solutions were added to the premoistened soil (to which water was previous added considering the 50% of the maximum WHC) and mixed manually [23]. The replicates were mixed individually. Tests started 1 d after soil spiking.

Table 3. Correspondence between the nominal concentrations of polystyrene nanoplastics (NPls) and the nominal concentrations of tween 20 and sodium azide (NaN_3).

NPls Nominal Concentration (mg/kg)	Tween 20 + NaN_3 Nominal Concentrations (mg/kg)
0.015	0.0029 + 0.00037
1.5	0.29 + 0.037
15	2.9 + 0.37
150	29.1 + 3.7
300	58.3 + 7.4
450	87.4 + 11.1
600	116.5 + 14.8
900	174.8 + 22.2
1200	233 + 29.6
1500	291.3 + 37

2.3.2. Enchytraeid Reproduction Test (ERT) Procedures

The ERT procedures followed the Organisation for Economic Co-operation and Development (OECD) guideline [23]. In short, 10 adult enchytraeids with well-developed clitellum and similar size were introduced in each test vessel containing 20 g of moist soil and 11 mg of food (autoclaved ground oats). The test ran for 21 d at 20 ± 1 °C and a 16 h:8 h (light: dark) photoperiod, with intermediate sampling points at 7 and 14 d. During the test, food (11 mg) and water content (based on weight loss) were replenished weekly. Seven replicates per experimental condition ($n = 7$) were used. An additional replicate per condition (without organisms) was prepared to measure the pH values. At the end of the test period, the organisms were fixed with ethanol and stained with 1% Bengal rose in ethanol. After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5 and 0.3 mm) to separate the organisms from most of the soil and facilitate counting. Adult and juvenile enchytraeids were counted using a stereomicroscope, and survival and reproduction were evaluated.

2.3.3. Avoidance Test Procedures

The avoidance test was performed following the earthworm avoidance test ISO guideline [24] with adaptations as described in Bicho et al. [25]. In short, containers (2.5 × 6.5 ø cm) with one removable divider were used; each replicate contained 50 g of soil (25 g each side), 25 g of control soil and 25 g of spiked soil. Following this, the wall was gently removed and 10 adult enchytraeids (with well-developed clitellum) were placed on the contact line of the soils. Boxes were covered with a lid (containing small holes) and kept, for 48 h, at 20 ± 1 °C and a photoperiod of 16 h:8 h (light: dark). Five replicates (n = 5) per experimental condition were used. An additional replicate per condition (without organisms) was prepared to measure the pH values. At the test end, the divider was again introduced in the separation line between the two soils (control and spiked soil) and each side of the box was independently searched for enchytraeids.

2.4. Exposure via Water

The test procedures were done based on the Daphnia acute immobilization test [22] as described by Römbke and Knacker [26] for *Enchytraeus albidus* and as reproduced in [27–29]. The exposure via

water was performed to compare the results from exposure via water versus via soil. Indeed, many terrestrial enchytraeids can survive in water [26]. The following nominal concentrations: 0.015, 1.5, 150 and 1500 mg NPls/L were tested in ISO water. A dispersant control (with tween 20 + NaN_3) was also performed, adding the same volume of the dispersant aqueous solution as used with the highest concentration of NPls (1500 mg/L). The exposure was performed in glass petri dishes containing 8 mL of ISO water each, with 10 organisms per petri dish and 3 replicates per experimental condition. The test conditions were 20 ± 1 °C with a 16 h:8 h (light:dark) photoperiod. The test was performed using adult enchytraeids with well-developed clitellum and similar size, ran for 5 d and survival was assessed at each 24 h. Organisms were considered dead when not responding to any mechanical stimulus. Observations were performed using a stereo microscope and it was captured in photographs (Dinocapture 2.0) (Almere, The Netherlands) (Figure S1).

2.5. Data Analysis

Graphics and statistical analysis were done applying the Sigma Plot 12.5 software package (Munich, Germany). Shapiro–Wilk and Levene's test were applied to evaluate the normality and homoscedasticity of data, respectively. To assess differences between control and NPls treatments, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test was employed. When data failed the normality and homoscedasticity tests, a non-parametric Kruskal–Wallis' test was done. Differences between control and dispersant control were carried out using a Student *t*-test. Significant differences were accepted for a significance level of $p < 0.05$. Toxicity Relationship Analysis Program (TRAP) 1.22 (Washington, DC, USA) was used to fit data in adequate models and to calculate the Effect Concentrations (ECx).

The avoidance response expresses the percentage of affected organisms (specifically those which avoided the spiked soil) and was determined according the earthworm avoidance test guideline [24]. Percentage of avoidance per treatment was calculated as *A*:

$$A = \frac{C - T}{N} \times 100$$

C: number of organisms in control soil; *T*: number of organisms on spiked soil; *N*: total number of organisms used per replicate.

Positive values reveal avoidance behaviour and negative values indicate a non-response or attraction to the contaminant (in this case, the NPls). Percentages of avoidance (A) ≥ 80% suggest limited habitat function [24].

3. Results

3.1. Characterization of Nanoplastics (NPls)

The characterization of NPls stock dispersion and diluted in ISO water, at the different concentrations used in the tests, showed that the particles presented an average hydrodynamic size of 69 nm, PDI of 0.032 and a ZP of around −23 mV. The obtained UV-Vis spectrum revealed that the maximum absorption peak of NPls was at around 520 nm (Figure 1a) and the FTIR spectrum showed the typical vibrational bands of polystyrene (Figure 1b).

3.2. Exposure via Soil

3.2.1. Enchytraeid Reproduction Test (ERT)

For the ERT, there were no significant changes in soil pH within the experimental conditions or over the test duration (21 d). NPls dispersion significantly reduced the survival of the organisms at 1500 mg/kg ($p < 0.05$; Figure 2) and significantly decreased the reproduction at 1200 and 1500 mg/kg ($p < 0.05$; Figure 2). Table S1 shows the average number of *E. crypticus* juveniles counted after 21 d

exposure to NPls dispersion and the average number of organisms counted in the control group. The calculated Median Effective Concentrations (EC 50) for survival and reproduction of *Enchytraeus crypticus* were around 1541.40 and 1321.10 mg/kg, respectively (Table S2).

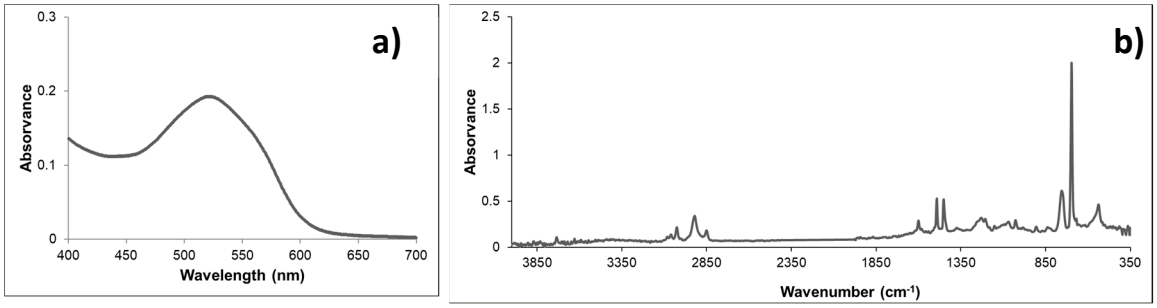


Figure 1. UV–Vis (**a**) and Fourier transform infrared spectroscopy (**b**) spectra of polystyrene nanoplastics dispersion.

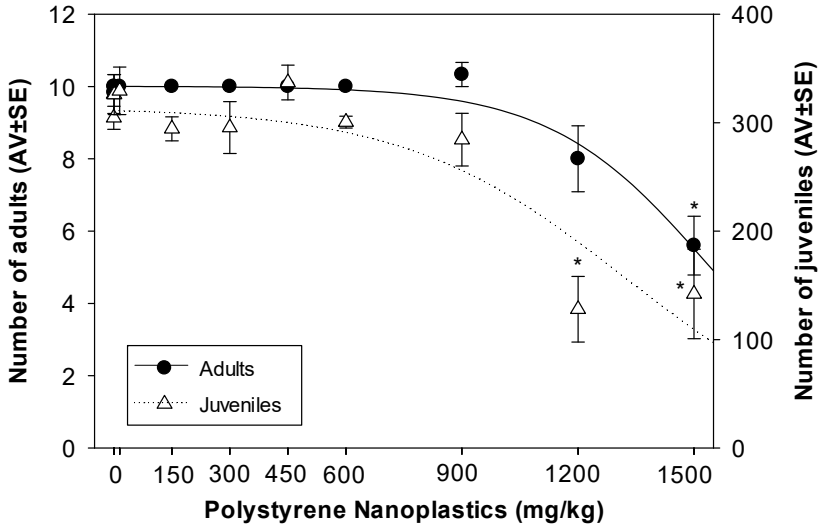


Figure 2. Enchytraeid reproduction test data in terms of survival (number of adults) and reproduction (number of juveniles) of *Enchytraeus crypticus* after 21 days exposed to the polystyrene nanoplastics (NPls) dispersion in LUFA 2.2 soil. Data are expressed as average value (AV) ± standard error (SE). Lines represent the models fit to data. * Significant differences to control ($p < 0.05$).

The dispersant solution—tween 20 + NaN_3—significantly reduced the survival of the organisms at the experimental conditions: 233 + 29.6 and 291.3 + 37 mg/kg ($p < 0.05$; Figure 3a) and significantly decreased the reproduction at 174.8 + 22.2, 233 + 29.6 and 291.3 + 37 mg/kg ($p < 0.05$; Figure 3). Table S3 shows the average number of *E. crypticus* juveniles counted after 21 d exposed to tween 20 + NaN_3 and the average number of organisms counted in the control group. It was not possible to calculate the ECx for the dispersant solution due to the concomitant presence of the two compounds, consequently this did not allow to discriminate the ECx for each one.

3.2.2. Avoidance Test

There were no significant changes in soil pH within the test conditions or over the test duration (48 h). Moreover, there were no significant differences between the control and dispersant control ($p > 0.05$). Therefore, the differences were assessed between treatments and the control group. There was a tendency of the organisms to avoid the NPls dispersion contaminated soils between 0.015 to 150 mg/kg, with this response being significantly different from the control at the lowest tested concentration (0.015 mg/kg)—Figure 4.

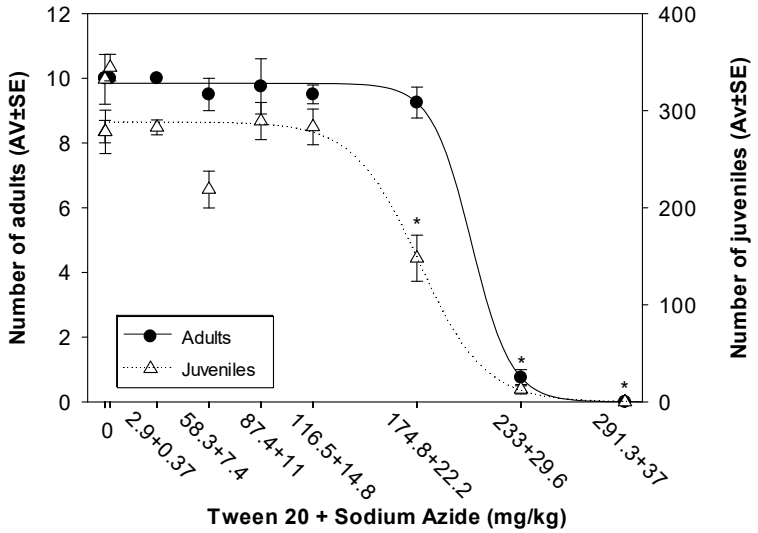


Figure 3. Enchytraeid reproduction test data in terms of survival (number of adults) and reproduction (number of juveniles) of *Enchytraeus crypticus* after 21 days exposed to the dispersant solution—tween 20 and sodium azide—in LUFA 2.2 soil. Data are expressed as average value (AV) ± standard error (SE). Lines represent the models fit to data. * Significant differences to control ($p < 0.05$).

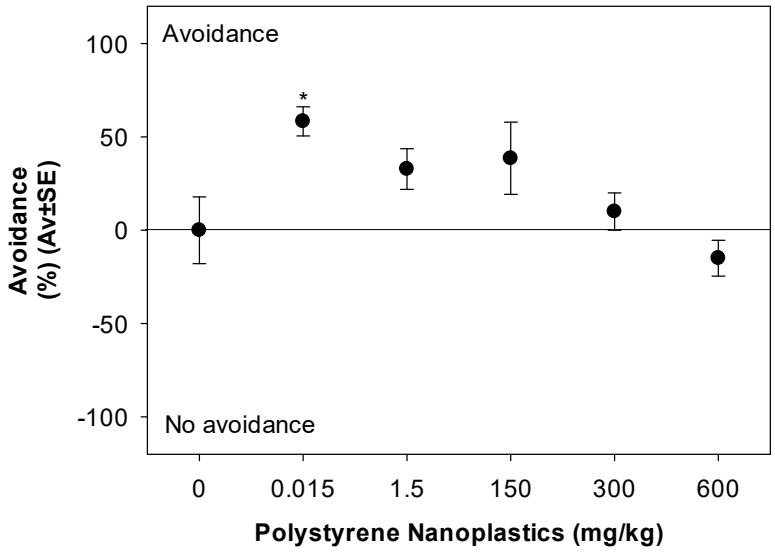


Figure 4. Avoidance responses of *Enchytraeus crypticus* after 48 h exposure to polystyrene nanoplastics (NPls) dispersion. Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

3.3. Exposure via Water

After 24 h, 100% of mortality was observed at the conditions: dispersant control, 150 and 1500 mg NPls/L. The other conditions (0.015, 1.5 and 15 mg NPls/L) caused no mortality during the time of the exposure. The macroscopic visualization showed what seemed to be an adsorption of the red dyed particles to the clitellum area and NPls inside the organisms after 5 d of exposure (Figure S1). However, dyed particles around the organisms (mostly in the clitellum area or cocoon) and inside the organisms were seen at 24 h until the end of the exposure. Based on the red colour observed around and inside the organisms, it seems that the NPls amount increased with the increase of NPls concentration.

4. Discussion

The physicochemical characterization of NPls is crucial to: i) understand the correlation between the NPls biological effects and their characteristics and ii) study the possible characteristics alterations and behaviour of NPls on the environmental media. The assessment of the physicochemical behaviour of NPls in environmental matrices, such as soil, is currently a challenge due to a lack of adequate protocols, expensive techniques and the difficulty of developing suitable methods for better characterization [21,30]. In the present study, the characterization of NPls in the stock dispersion and diluted in ISO water showed that particles had a negative surface charge, the expected size and a low PDI, indicating that the NPls dispersions were highly monodisperse. This characterization showed the stability of the NPls in ISO water. However, some aggregates/agglomerates were seen in the exposure with organisms as depicted in the obtained photos of *E. crypticus* exposed to NPls via ISO water (supplementary materials). This can be related to the exudates produced by the organisms during the exposure that can interact with the NPls.

With the fragmentation, plastics acquire new physicochemical characteristics that enhance their potential interaction with organisms causing direct and/or indirect toxicity [4]. Due to the size of MPls/NPls, soil fauna may consume them when feeding [6]. Decreased reproduction and growth, and several other harmful effects have been previously detected in soil organisms exposed to MPls and NPls [5,6,15,16]. In our study, only the two highest tested concentrations of NPls dispersion had significant effects on the organisms survival and/or reproduction. However, these effects may be mainly associated with the toxicity of tween 20 and NaN_3 present in the NPls dispersion. As tested in the present study, in general, ecotoxicological studies test commercially available formulations of polystyrene MPls and NPls, containing various preservatives, antimicrobials or surfactants, such as NaN_3, tween 20 and sodium dodecyl sulfate [31]. As previously reported, the presence of these compounds may introduce artefacts in the toxicity evaluations [31], corroborating the data found in our research. The results from an acute toxicity test on *Daphnia magna*, using commercial polystyrene NPls (20 and 200 nm) dispersion containing 2 mM NaN_3, showed that the acute toxicity of the commercial formulation of polystyrene NPls was mainly associated with NaN_3 and not with NPls themselves. In our study, this is also shown by the exposure via ISO water where the dispersion of 1500 mg NPls/L induced 100% of organisms mortality due to the presence of tween 20 and NaN_3. These results show the importance of removing the preservatives, antimicrobials or surfactants present in the NPls dispersions to know only the effects induced by NPls. An alternative could be via centrifugation of the NPls dispersions, without compromising particle characteristics, to remove these compounds, and can be performed in further ecotoxicity tests to assess the specific effects of NPls. On the other hand, our study highlighted the importance of considering the toxicity of other compounds present in commercial plastics that can induce harmful effects on the organisms and the interaction of NPls with them inducing dissimilar effects than those expected.

An interesting result was found in this work, it seems that NPls alleviated the effects of tween 20 and NaN_3. Dispersion of 900 mg/kg of NPls containing 174.8 mg/kg of tween 20 and 22.2 mg/kg of NaN_3 had no effect in terms of organism reproduction. However, exposures to the dispersant solution—tween 20 + NaN_3—at the same concentrations (174.8 + 22.2 mg/kg) decreased the reproduction of *E. crypticus*. The mechanisms behind the altered toxicity of tween 20 and NaN_3 by the presence of NPls, found in the present study through the exposure via soil, should be further investigated but may be related with incorporation rates, sorption ability, cellular defence mechanisms and different modes of action. NPls are capable of adsorbing to biomolecules (such as proteins, lipids and metabolites) present in the organisms [32,33]. This adsorption may alter the dynamics of the surface ionic charges in the environmental context [34,35] and, ultimately, resulting in altered toxicity of the complex containing NPls and adsorbed elements [36]. The possible absorption of tween 20 and NaN_3 to NPls may result in less bioavailability of these compounds to the organisms and consequently less interaction, resulting in less pronounced effects on organisms reproduction when compared with the effects induced by tween 20 and NaN_3 single exposures.

Organisms tended to decrease the avoidance responses with the NPls concentration increase. Indeed, the organisms' avoidance responses were only significant at the lowest tested concentration (0.015 mg/kg). The lower aggregation/agglomeration processes of NPls at low concentrations when compared with high concentrations can explain the response pattern observed. As already reported, aggregation/agglomeration processes are likely to increase with the increase in the number of particles per volume [37]. At low concentrations, NPls can maintain their sizes, presenting higher likelihood to be detected by the organisms inducing protective mechanisms as the avoidance responses detected. Nonetheless, the present findings highlight the importance of studying the effects of NPls at low concentrations as previously shown for nanomaterials [38]. On the other hand, with increasing concentrations, more NPls are present in the soil, reacting with the organisms and may induce a negative effect (e.g., a neurotoxic effect). This neurotoxic effect can induce a non-response in the organisms, not being possible for them to avoid the contaminated soil. Indeed, Bicho et al. [25] reported that the non-avoidance behaviour of *E. crypticus* might be associated with the gamma-aminobutyric acid (GABA) system. The up-regulation of the GABA is known to trigger anaesthetic effects [39], hence this may at least partially explain the obtained results. Other invertebrates, such as nematodes—*Caenorhabditis elegans* (live in the interstitial water of soil)—altered their locomotor behaviour after polystyrene (nano)microplastics exposure (3 d to 1 mg/L) [40]. Despite being related to an aquatic vertebrate, behavioural alterations were also detected in locomotion, aggressiveness, shoal formation and predator avoidance of *Danio rerio* adults (7 d exposure to 1.5 mg/L polystyrene NPls) [41]. Overall, this can indicate the ecological impact of NPls in soil: organisms are not efficiently able to escape to non-contaminated soils, become intoxicated, and this can cause impact in other biological responses. The calculated percentages of avoidance (the highest was A = 58.3% for the condition 0.015 mg NPls/kg) were not equal or higher than 80%, which can suggest that the habitat function will not be compromised after the exposure to the tested concentrations of NPls.

A NPls dispersion toxicity dependent on the medium of exposure (i.e., soil versus water) was demonstrated in the present study. Indeed, 150 mg NPls/Kg via LUFA soil induced no effect in terms of the survival of the organisms after 21 d, although 150 mg NPls/L via ISO water induced 100% of organisms mortality after 5 d. Toxicity via soil was comparatively lower than via water exposure, as previously reported for different environmental contaminants [26]. We hypothesize that this could be due to the interaction of NPls with the soil particles, decreasing the bioavailability of NPls and consequently inducing less effect on the organisms. Previous studies already showed that soil components, such as soil mineralogy, clay content (soil texture) and the amount of organic matter, can modulate the toxicity of the nanomaterials [27,30].

Further studies to evaluate the effects of NPls, alone or associated with other compounds, assessing different endpoints, such as gene expression and biochemical changes for neurotoxicity, inflammatory and oxidative stress responses are very welcome. Indeed, effects at sub-individual levels have been reported after NPls exposure in aquatic organisms [11,42]. As NPls are ultimately the final particle obtained during the plastic degradation and disintegration, more investigation on its uptake and toxicity mechanisms in soil organisms is essential, considering their effects alone and when in the presence of other compounds.

An important aspect to consider is the suitability of current guidelines to assess the hazard of NPls. As discussed in Amorim et al. [43] alternatives for highly durable materials, like plastics, can be studied using i) longer exposure durations and ii) after prior ageing and weathering of materials. These concerns have common aspects with the existing for PBTs (persistent, bioaccumulative and toxic) and vPvBs (very persistent and very bioaccumulative) compounds.

5. Conclusions

The highest tested concentrations of polystyrene NPls dispersion (1200 and 1500 mg NPls/kg), 21 d exposure via soil, had an impact on the *E. crypticus* survival and reproduction (decreased). On the other hand, the exposure via water showed that polystyrene NPls dispersion induced 100% of mortality

at concentrations ≥ 150 mg NPls/L. However, these effects seem mainly associated with tween 20 and NaN_3 present in the polystyrene NPls dispersion and not with NPls themselves. Nevertheless, there was a tendency of the organisms to avoid the polystyrene NPls dispersion spiked soils, being this response relevant at the lowest tested concentration (0.015 mg NPls/kg). Indeed, organisms decreased the avoidance responses with the polystyrene NPls concentration increase. A NPls dispersion toxicity dependent on the medium of exposure (soil versus water) and NPls concentration was found in our study. The obtained results show that there is an urgent requirement to focus on research about this topic, and to provide more knowledge about environmental behaviour, as well as ecotoxicological data about NPls (exposed alone and with other compounds) in terrestrial ecosystems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/21/7674/s1, Figure S1: Representative images of *Enchytraeus crypticus* adults when exposed during 5 days in ISO water to polystyrene nanoplastics (NPls). Black arrows indicate NPls agglomeration/aggregation or adsorption in the clitellum/cocoon area, Table S1: Number of *Enchytraeus crypticus* juveniles counted after 21 d exposure to polystyrene nanoplastics (NPls) in LUFA 2.2 soil. Results are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$), Table S2: Number of *Enchytraeus crypticus* juveniles counted after 21 d exposure to the dispersant—tween 20 and sodium azide (NaN3)—in LUFA 2.2 soil. Results are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$), Table S3: Effect Concentrations (ECx), applying the 2-parameters Logistic model, for survival and reproduction of *Enchytraeus crypticus* after 21 d exposure to polystyrene nanoplastics (NPls) in LUFA 2.2 soil. EC 20, 50, 80: Concentration that causes 20, 50 and 80% of effect, respectively. Results are presented as estimated value ± standard error.

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