Plastic pollution – A case study with *Enchytraeus crypticus* – From micro-to nanoplastics[☆]

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A B S T R A C T

The concern about microplastic (a group of polymers) in the environment may cause us to overlook a more substantial problem: microplastics will fragment into nanoplastics. This fragmentation will lead to a high number of nanoplastics particles. Such nanoplastic can be taken up by cells, as opposed to microscale particles that are either not or to much less extend taken up. Fragmentation into nano will also release materials previously safely embedded in the polymer. We here present results from 25 OECD/ISO *in vivo* hazard tests, and beyond, e.g. extended exposure duration, with *Enchytraeus crypticus*, using pristine nano-scale materials (NMs) [CuO, Fe_2O_3, Organic Pigment, MWCNT], fragmented products (polymers) with these NMs embedded in the matrices (FP_NM), and fragmented polymers without NMs (FP) [covering the 4 major plastic types: Acrylic, Polyethylene, Polypropylene and Epoxy]. For example, MWCNTs induced a highly significant population decrease after extended period of 60 days, despite having no impact after 28 days' exposure, the standard OECD duration. We conclude, that the standard tests were not suitable to evaluate hazards of these plastic fragments, weathering/ageing of materials is recommended, and extension of test duration can add value to the testing of NMs. We must refocus the concern to testing with polymers (not only "plastics"), from micro-to nano-polymers, and from aquatic to terrestrial environments.

Author statement

1. Introduction

There is considerable attention regarding microplastics in nature, both in media and science, especially with focus on the aquatic environment (Haward, 2018; Jia et al., 2019; Koelmans et al., 2017). Although it is recognized that nanoplastic is of concern, the majority of publications is mainly dealing with microplastic (Chae and An, 2018; Piehl et al., 2018; Selonen et al., 2019; Wang et al.,

2019a,b). The size range is found variable and still lacks consensus (Hartmann et al., 2019) (e.g. nanoplastics <100 nm; microplastics >100 nm to <100–5000 μm). In fact, the environmental issues arising from the abundance of micro-scale plastic particles (World et al., 2018) may represent only the tip of the iceberg because most microplastics will, when they degrade, inevitably become nanoforms, before they (maybe) totally disappear (Fig. 1A).

It is well established that nano-scale particles are taken up much more readily and rapidly than their microscale counterparts (Paul et al., 2013; Richards and Endres, 2017). In fact, whereas the nanoscale particles can cross cell-membranes only the smallest microscale particles will (Fig. 1B). These two facts, i.e. that plastic will end up as nanoplastic and that cellular uptake almost exclusively happen at the nano-scale advocate that we must change focus – the focus should be on potential effects of nanoplastics.

On a global scale, if we do not act to reduce humanity's production of plastic waste, the number of nanoplastic particles on Earth may be as high as 10^{32} - 10^{35} by 2050 (see calculation in box below). It is sometimes difficult to grasp the scale of the issue with
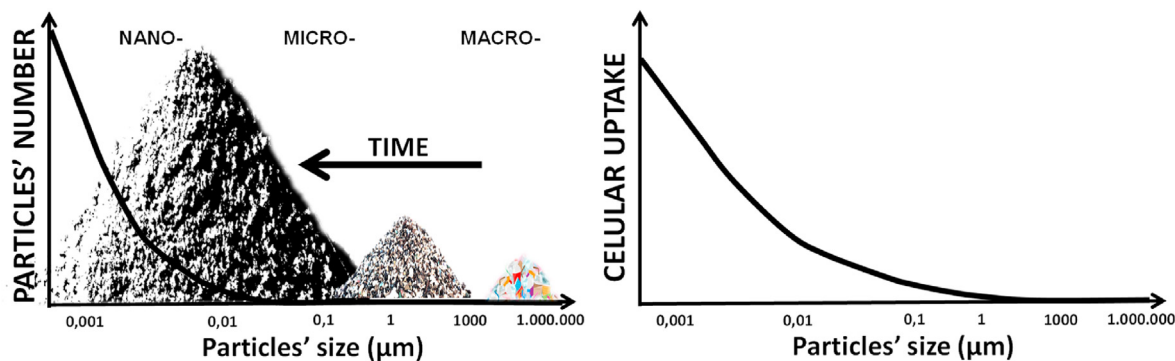

Fig. 1. Schematic representation of A) the gradual degradation of plastic materials occurs with time, from plastics (small coloured pile) to macro-meso-microplastics (medium pile) and finally nanoplastics (large pile), with the number of particles increasing and B) the relationship between particle size and possible cellular uptake: the smaller the size, the greater the uptake. The upper limit for uptake is reported in the lower μm diameter (Paul et al., 2013; Richards and Endres, 2017).

micro-nano-plastic, so to provide a bit of perspective of what such numbers is equivalent to, so for comparative purposes, there are only around 10^{24} stars in the universe (Cain, 2013). Even with the ongoing growth of the human population, the number of nanoplastic particles per human will be staggeringly high - approximately 10^{23} nanoplastic particles per human in 2050 (for comparison, the human body has only $3.7*10^{13}$ cells (Bianconi et al., 2013)).

"back of an envelope" calculation:

1. Predicted **plastic** production by 2050 ≈ 33000000000 tonnes (Rochman and Browne, 2013).
2. Average weight of a **plastic nanoparticle** (Writer, 2020):

   10 nm ≈ $3*10^{-22}$ kg, 50 nm ≈ $3*10^{-20}$ kg, and 100 nm ≈ $3*10^{-19}$ kg.

3. Number of nanoplastic particles of:

   10 nm: n ≈ $1*10^{35}$ particles.

   50 nm: n ≈ $1*10^{33}$ particles.

   100 nm: n ≈ $1*10^{32}$ particles.

   (Writer, 2020) assuming a density of 0.5 g/cm^3 [assuming a different density e.g. 0.9 the equivalent number would be $1*10^{28}$, $1*10^{26}$ and $1*10^{25}$].

Obviously, societies around the world are and will be doing something about this (Haward, 2018; Jia et al., 2019). However, the scale of the problem is incredible [please note, this does not mean it is directly related to hazard (Völker et al., 2020)]. Even with action being taken (Winnie W. Y. Lau et al., 2020), the $31.9*10^6$ tons/per year of mismanaged plastic waste leaking into the environment (Rochman, 2018) is equivalent to 10^{29}-10^{32} nanoplastic particles.

The fragmentation of plastics into nanoscale particles will enable a different and far wider environmental distribution because smaller and lighter particles are more readily spread (Ostle et al., 2019). For example, flow of micro-/nano-plastic particles to terrestrial ecosystems is predicted to be 40 times larger than into aquatic ecosystems (Kawecki and Nowack, 2019; Mitrano et al., 2019). Despite this, little is known about the effects of

nanoplastics in the terrestrial environment (ECHA, 2019; Rillig and Bonkowski, 2018; Rodriguez-Seijo et al., 2017; Zhang et al., 2018); (Xu et al., 2020).

For society, regulatory environmental hazard assessments of (pre)marketed products such as plastics are largely based on hazard assessments of their individual chemical constituents or active ingredients. There is an ongoing debate about how novel combined materials such as nanomaterials and plastics should be covered by regulation and how best to assess their impact over their complete life cycle (ECHA, 2019; Gouin et al., 2019; OECD, 2012a; Scott-Fordsmand et al., 2017a,b). While polymers [all plastics are polymers, but not all polymers are plastics] are widely used they are generally exempt from registration, however they can be treated as microplastics "… where they meet the specific conditions that identify them as being microplastics and where their use will result in releases of microplastics to the environment" (ECHA, 2019). A recent proposal argues that nano- and microplastics should in some cases be treated as Persistent, Bioaccumulative, and Toxic (PBT) or very persistent and very bioaccumulative (vPvB) substances (ECHA, 2019). The time-frame of a plastic's degradation, i.e. its persistence, obviously depends on both environmental factors and the polymer's properties (Amorim et al., 2018; Neubauer et al., 2017; Wohlleben et al., 2017). The more "durable" the polymer, the longer its degradation will take (GESAMP (Group of Experts on the Scientific Aspects of Marine Environmental Protection), 2015), which is why we belive the wording should shift from micro- and nanoplastics to micro-and nano-polymers. Again, simple logic suggests that meaningful hazard testing of products such as polymers containing embedded engineered nanomaterials is only possible if we know how long it takes for the polymer to degrade before the embedded nanomaterial is released (Scott-Fordsmand et al., 2017a,b). Only then can we assess the hazard that exists in the worst-case scenario (Gómez and Michel, 2013; Irizar et al., 2018). To obtain the necessary information, hazard-testing strategies could be adapted to include extended test durations and/or fragmentation-weathering of the test material prior to testing (Amorim et al., 2020). Extended hazard testing has proven to be beneficial in some cases (Bicho et al., 2017a, 2017b; Mendes et al., 2018; Ribeiro et al., 2018), and the weathering of nanomaterial-containing polymers has been suggested, demonstrated (Nowack et al., 2016) and performed (Neubauer et al., 2017).

In this study we set out to identify better safety testing procedures for complex plastic-based materials, and through 25 OECD/ISO *in vivo* tests we investigated the effects of extended exposure times and prior fragmentation-weathering on the outcomes of safety tests using these materials. Tests involving extended exposures were readily performed, as were tests using fragmented

material. However, it was not possible to adequately mimic the effects of weathering; while small quantities of artificially weathered material could be generated, it was impossible to generate enough material with sufficient uniformity for testing. This was not due to a lack of expertise, because despite our participation in large European projects investigating the fates and effects of nanomaterial-embedded products, we were only able to obtain low mg to μg quantities of fragmented cryo-milled materials without weathering. We conducted the 25 OECD/ISO *in vivo* hazard tests using the worm *Enchytraeus crypticus*, because they are the most important organisms in many habitats, dominant both in biomass and abundance, e.g. abundance ranging between 10^2-10^5 per m^2 (Pelosi and Römbke, 2018). The polymer materials tested included organic and inorganic, as pristine nanomaterials (NM), fragmented products with embedded NMs (FP_NM) in polymer matrices, and the fragmented polymer matrices (FP_M) themselves. We also tested the importance of extending the test duration. To mimic real-world conditions, we used cryo-milled materials that mimic the debris formed by fragmentation and transformation of these materials during the use and disposal phases of their lifecycles. Below we report the results of these tests, discuss the practical challenges arising from our findings, and outline their implications.

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) (ISO, 2003; OECD, 2004) was used as the test species in this work. Cultures were kept in agar plates for several years at the University of Aveiro and synchronized cultures were prepared as described previously (Bicho et al., 2015). Briefly, mature adults with well-developed clitella were transferred to fresh agar plates to lay cocoons and then removed after 2 days. Synchronized juveniles between 17 and 19 days old were used in the tests.

2.2. Test media

The standard LUFA 2.2 natural soil (Speyer, Germany) was used as the test media. This soil has a pH of 5.5 (0.01 M CaCl2, ratio 1:5 w/ v), an organic matter content of 1.77 meq/100 g, a CEC (cation exchange capacity) of 10.1%, a WHC (water holding capacity) of 41.8%, and a grain size distribution of 7.3% clay, 13.8% silt, and 78.9% sand. For the tests, the soil was moistened to 50% of its WHC with distilled water.

2.3. Test materials, characterisation and spiking procedures

The tested materials included various nanomaterials (NMs) and real products containing NMs (see Table S1). The recommended testing approach (Nowack et al., 2016) was used; all nanomaterials were tested as synthesized, along with fragmented products containing embedded NMs and fragmented samples of the polymer matrices without embedded NMs. Details of the testing and characterization procedures are available elsewhere (Amorim et al., 2018).

FPs were prepared by cooling products to cryogenic temperatures (−196 °C, liquid N_2) to maximize their brittleness. All materials were milled with a Pallmann PPL18 unit (10,000 rpm, 92 m/s circumferential speed). To generate smaller fragments, some of the materials were treated with a second mill (Hosokawa AFG100) that uses three impacting gas jets at pressures of 6 bar with a flux of 80 m^3/h, pre-cooled to −30 °C. The material was accelerated by the gas jets and fragmented by particle-particle collisions. Only small fragments could escape via the rotor at the top of the mill, which rotated at 3800 rpm (10 m/s circumferential speed); larger fragments were returned to the collision zone. The milling was continued for 60 min. The size distribution of FPs was characterized by laser diffraction using a Mastersizer 3000 (Malvern). FPs were poured into water containing 0.5 g/l SDS (Sodiumdodecylsulfate) at a concentration of 1 mg/ml and then sonicated.

The materials (powders) were directly mixed with the dried soil following the recommendations for non-dispersible nanomaterials (OECD, 2012b). Materials obtained as dispersions in solution (Acryl and Acryl_CuONM) and water soluble species ($CuCl_2$ and $FeCl_3$) were applied as aqueous solutions on pre-moistened soil. The soil moisture was adjusted to 50% of the soil's maximum water holding capacity (maxWHC). Each replicate was spiked individually to ensure that the amount of added material remained consistent.

2.4. Test procedures

The standard guideline (ISO, 2003; OECD, 2004) was followed, with modifications as reported previously (Bicho et al., 2015). Briefly, 10 synchronized age organisms were placed in each test container (⊘4 cm) with 20 g of moist soil and a food supply (24 ± 1 mg, autoclaved rolled oats). Tests ran for 28 days at 20 °C with a 16:8 h photoperiod. Food (12 ± 1 mg) and water were replenished weekly. Four replicates were run for each treatment, plus one without organisms for measurements of abiotic factors (e.g. pH) and material characterization. Additional replicates were performed to provide controls and at selected material concentrations (see Table S2 for details of all 25 tests) to monitor survival after 7, 14, 21, 56, 74, and 84 days of exposure. For replicates to be run for more than 28 days, larger test containers (⊘5.5 cm) were used with 40 g of soil per replicate because of the gradual increase in the organisms' density. For these replicates, adults were carefully removed from the soil on day 28, after which the soil was left and water and food were replenished weekly. At the end of the test, to facilitate extraction of organisms from the soil and counting, replicates were fixed with 96% ethanol and Bengal rose (1% solution in ethanol). Samples were then sieved through three meshes (0.6, 0.2, 0.1 mm) to separate individuals from most of the soil and to facilitate counting using a stereo microscope. Endpoints included survival and reproduction (numbers of adults and juveniles, respectively).

2.5. Data analysis

Effect concentrations (ECx) were estimated by modelling data using threshold sigmoid 2 parameters regression models, as indicated in Table S3, using the Toxicity Relationship Analysis Program (Erickson, 2012) software. One-way analysis of variance (ANOVA) followed by Dunnett's comparison post-hoc test ($p < 0.05$) was used to evaluate differences between controls and treatments.

3. Results

Three materials − nanoscale CuO (CuO NM), organic pigment, and $CuCl_2$ - exhibited dose-dependent effects on *E. crypticus* survival and reproduction (Fig. 2; see also Fig. S1 for results obtained with $CuCl_2$ and $FeCl_3$). However, exposure to the fragmented plastic products containing these nanomaterials had no effect at the tested levels and durations of exposure.

The estimated effect concentrations (Table S2) showed that dose response models could be fitted for CuO NM, organic pigment, and $CuCl_2$ within the tested concentration ranges. To evaluate the effects of prolonged exposure, we tested exposure periods of 60 and 84 days to complement the standard 28 day tests (Fig. 3). This revealed that the effects of the fragmented products PP_MWCNT
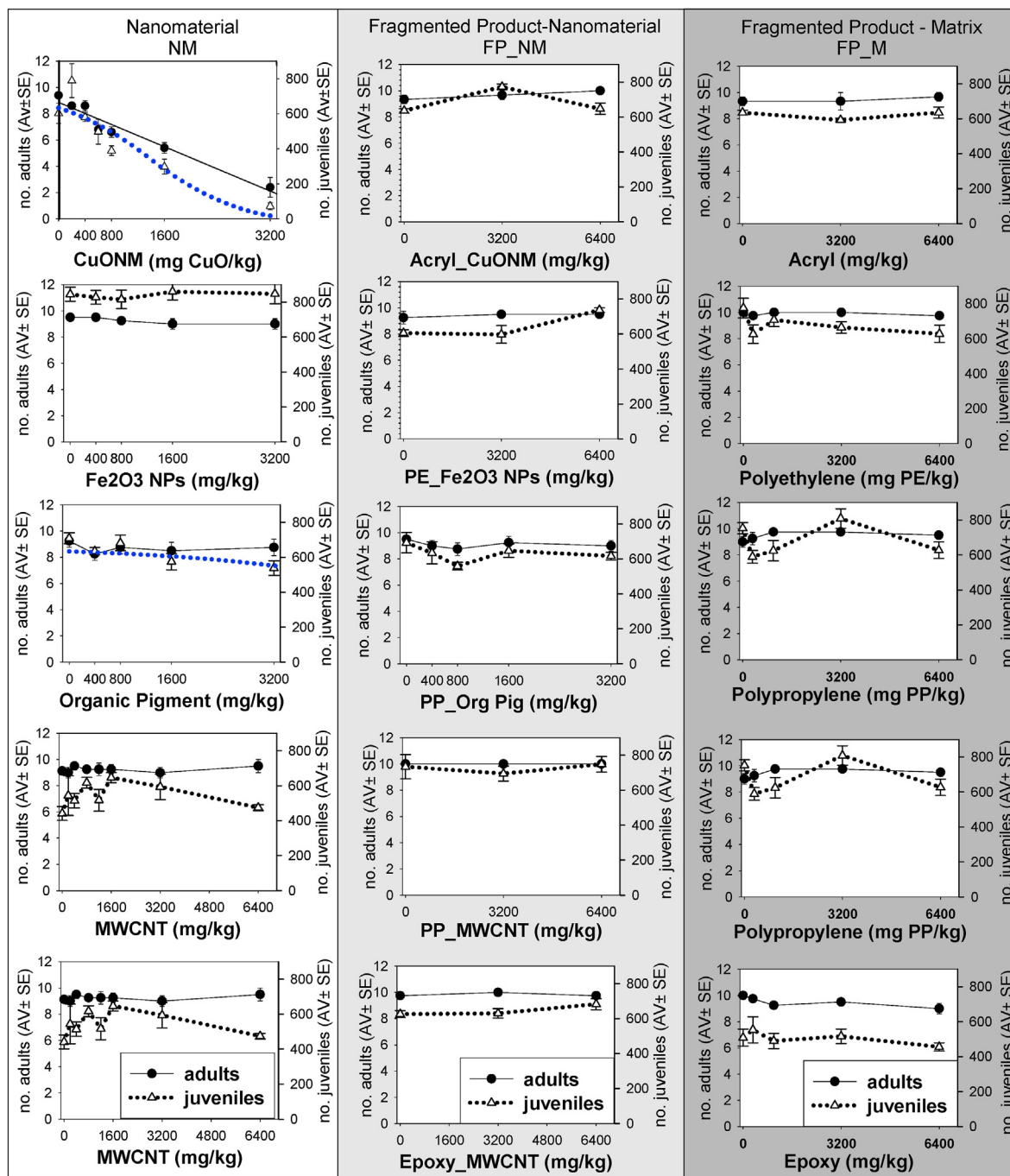
Fig. 2. Standard Enchytraeid Reproduction Tests as dose-response. Results showing the survival and reproduction of *Enchytraeus crypticus* exposed for 28 days to nanomaterials (NM), fragmented products (FP) with embedded NMs, and fragmented matrix products (FP_M) in LUFA 2.2 soil. The NMs included CuO, Fe$_2$O$_3$, an organic pigment, and Multi Walled Carbon Nanotubes (MWCNT). The FP_M included Epoxy, Polypropylene, Polyethylene, and Acrylate (mg FP/kg DW soil). All values are expressed as means $\pm$ standard error (Av $\pm$ SE). Blue dotted lines show the dose-response model fit to the data for CuO NM and organic pigment.

and PP_Org Pig increased over time.

The extended exposure tests revealed that long exposure increased the impact of certain NMs. For example, MWCNTs induced a highly significant population decrease after 60 days at the highest tested concentration ($F_2 = 42.5$, $p < 0.001$) despite having no significant effect after 28 days' exposure (Fig. 4). At 1600 mg MWCNT/kg soil a positive significant effect was observed ($F_2 = 42.5$, $p = 0.002$). Conversely, Fe$_2$O$_3$ NPs had no effect on the *E crypticus* population under any conditions.

The population growth curves (not shown) were well fitted by

van Bertalanffy logistic models with density inhibition after 84 days (see Figs. S2 and S3).

4. Discussion

The results presented here show that standard toxicity tests are not fully fitted for assessing the hazards of plastic (polymer) nanoscale particles but are in some case sufficient to identify toxic effects of polymer additive nanomaterials in their pristine (i.e. matrix-free) state. No toxicity was detected in either standard or
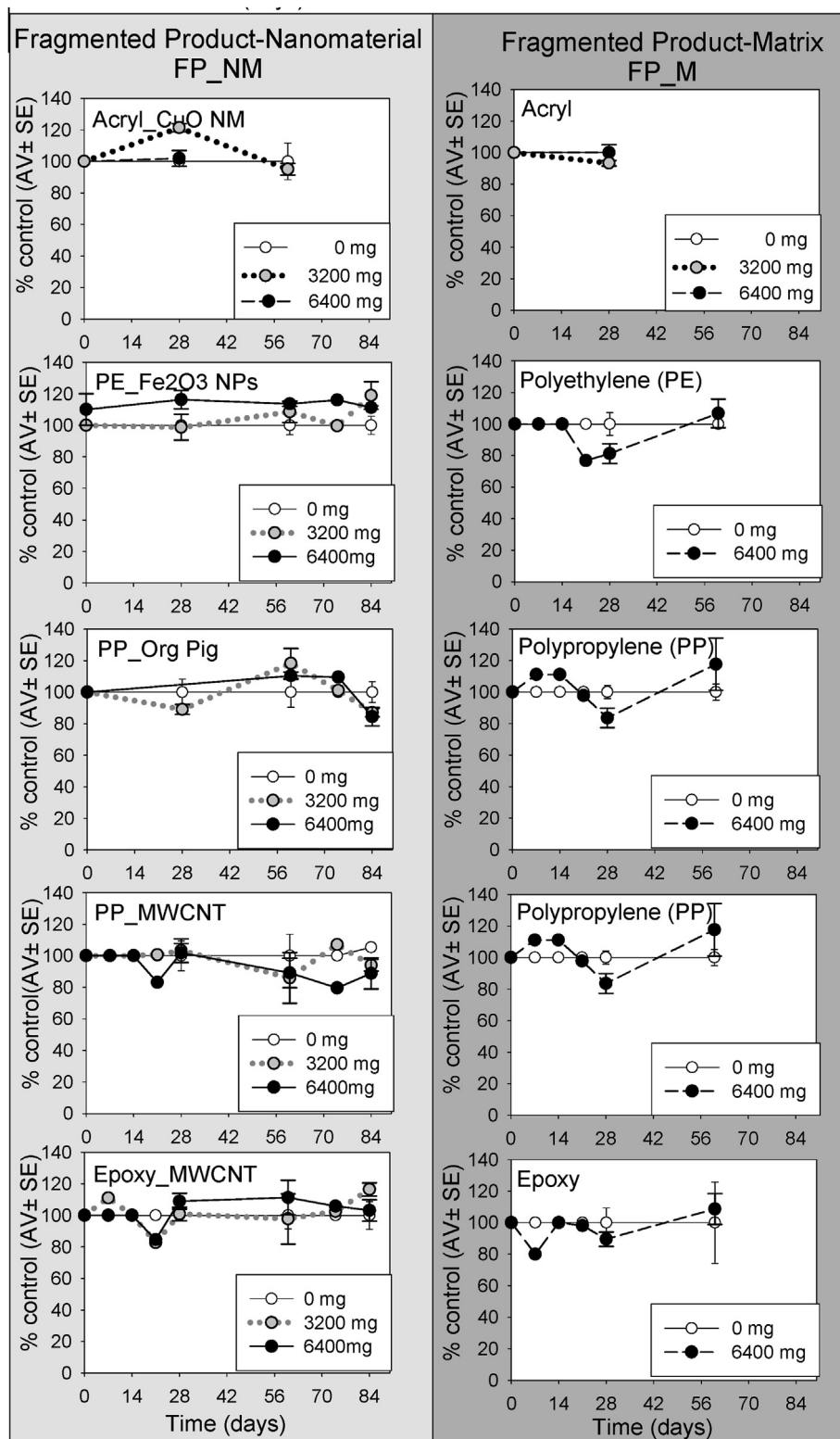
Fig. 3. Enchytraeid Reproduction Tests monitored in a time series. Results showing the total number of *Enchytraeus crypticus* individuals (surviving adults and juveniles) after prolonged exposure to the tested materials. Numbers of living individuals were counted after 7, 14, 21, 28, 56, 74 and 84 days' exposure to fragmented products (FPs) with embedded NMs (FP_NM) and fragmented matrix products (FPs) (M: Acrylic, Polyethylene, Polypropylene and Epoxy) in LUFA 2.2 soil. All quoted values are means ± standard error (Av ± SE), expressed as percentages of the relevant control result.
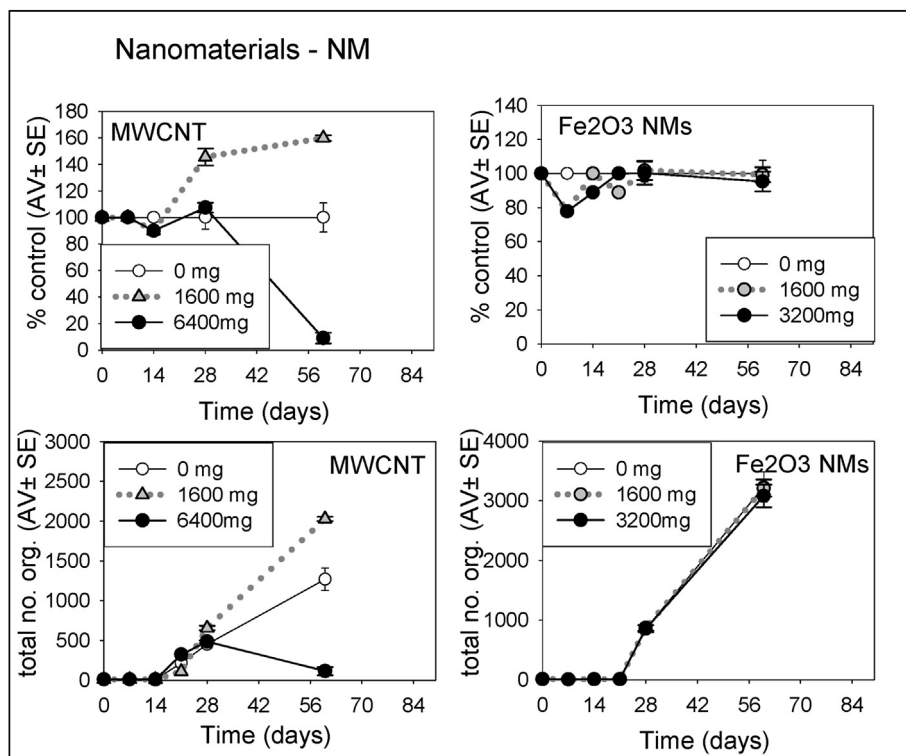
Fig. 4. Enchytraeid Reproduction Tests monitored in a time series. Results showing the total number (or the total number as a percentage of the control) of *Enchytraeus crypticus* individuals (survival plus reproduction) after prolonged exposure to the tested materials. Numbers of living individuals were counted after 0, 7, 14, 21, 28, 56, 74 and 84 days' exposure to two nanomaterials (NMs): MWCNT and Fe_2O_3 NPs. All values are expressed as means ± standard error (Av ± SE).

extended tests of composite materials containing these additives embedded in various polymers (or the polymers without additives). This was probably due to insufficient fragmentation and thus a lack of exposure to the embedded nanomaterials.

We saw that when testing pristine nanomaterials, the test duration should be longer than that specified for conventional chemical substances in order to properly assess the hazard they pose. Increasing the exposure time to 60 days (from the standard 28 days) revealed that MWCNTs had a clear toxic effect at the highest tested concentration. However, a positive effect on survival was observed at lower MWCNT concentrations (up to 1600 mg/kg soil), possibly because of the system's increased carbon content and direct toxicity was lower. No such effects, positive or negative, were observed for Fe_2O_3 NMs, independently of the duration of exposure. Nevertheless, it appears that increasing exposure times by a factor of 2–3 or even more may be beneficial, without greatly increasing the cost of testing, depending on the length of the test organism's life cycle and on the plastic type being tested. Several other studies have similarly demonstrated the importance of extended exposure times when testing nanomaterials such as WCCo NMs (Ribeiro et al., 2018) and CuO NM (in both single-species and multi-species tests) (Bicho et al., 2017a)' (Mendes et al., 2018).

Pure polymers and composite materials with nanomaterials embedded in polymers exhibited no detectable toxicity in the standard test, probably due to a lack of exposure to nano-scaled fragments or released nanomaterials. Attempts to account for the delayed release of these products by tripling the exposure duration did not change this outcome and may not be sufficient for meaningful hazard assessment. In tests with a prolonged exposure time (84 days), the growth of the *E. crypticus* population fitted the standard logistic van Bertalanffy growth curves, with only minor differences between populations exposed to different materials at

various concentrations. Test durations greater than 84 days cannot be recommended for very persistent (vP) materials with the current testing system because the population density increases over time and eventually becomes a confounding factor (Gonçalves et al., 2017; Menezes-Oliveira et al., 2013). These observations support the argument that polymers/plastics with embedded nanomaterials should be classified as PBT or vPvB. A better testing strategy may be to artificially age the materials in the exposure media, e.g. by using artificial weathering methods (Nowack et al., 2016)' (Neubauer et al., 2017) or by direct aging in the exposure media (in this case soil) with and without organisms (Dawson et al., 2018; Gouin et al., 2019; Irizar et al., 2018; Zhu et al., 2018). Despite its appeal in terms of standardization, our experience is that artificial weathering makes it difficult (if not impossible) to obtain sufficient weathered material with an adequately homogeneous nano-scaled distribution for use in environmental hazard testing. The alternative of ageing the plastic in soils (or sludge) may seem straightforward, but because it is almost impossible to directly identify the polymers in the complex media this is not as trivial as it might seem (Oliveira and Almeida, 2019; Prata et al., 2019). To enable a better detection, it has recently been suggested to dope the plastic with a tracer metal, but this is mainly a solution for experimental testing (Mitrano et al., 2019). To determine the extent of weathering during these processes, one must know the degradation pattern of the polymer being studied outside and inside organisms. We have previously studied polymer degradation in soil and sludge (Irizar et al., 2018), revealing that the degradation process depends on the soil type and whether or not sludge treatment was performed. Other studies (Chinaglia et al., 2018) have shown that degradation rates depend strongly on the composition and surface area of the studied samples. Finally some studies have shown that micro-can become nano-inside the organisms (Dawson et al., 2018). Therefore, rates of micro-plolymer

degradation in hazard tests could vary widely simply because of variation in particle size within the samples, which is why a homogeneous particle size distribution is important in standard tests. Moreover, since we tested mainly micro-scale materials (it was impossible to grind sufficient quantities of adequately uniform nanoscale materials), we would have had to determine at what point the materials were degraded to nanoscale and when the matrix was completely degraded to see whether their composition or structure (and thus, potentially, their toxicity) had changed (Scott-Fordsmand et al., 2017a,b).

Overall, the results presented here support the recently proposed new REACH (ECHA, 2019) restrictions, which state that micro- and nano-scaled plastics behave like PBTs and vPvBs even though they have no toxic effects in the standard test [it should be noted that the REACH proposal only targets deliberately manufactured and intentionally added micro- and nanoplastics]. Clearly, in cases where the additive is known to be toxic (Zimmermann et al., 2020) but no effect is observed in the standard test, the composite material must be a PBT or vPvB. If the additive is not known to have a toxic effect, we should probably still consider this paradigm because aside from the uncertainty about the additive's actual toxicity, it is as mentioned earlier well established that nanoscale particles are readily taken up by organisms, unlike most microscale particles. Finally, as outlined previously (Scott-Fordsmand et al., 2017a,b), there are several other challenges to overcome when testing such fragments, including *in situ* characterization in the complex exposure media, identifying reliable methods for estimating uptake, and potential changes in the properties of the media (Araujo et al., 2018; Da Costa et al., 2019; Petersen et al., 2019; Shim et al., 2017).

5. Conclusions

Standard guidelines do not seem fit for purpose within testing of materials such as plastics, Lessons learned from the adaptations of guidelines to assess hazards of NMs should be taken as certain NMs specific aspects apply to micro-nano-plastics. Some practical testing solutions envisage to include testing of weathered/aged materials and the extension of test duration, although both need careful considerations and may be plastic type dependent. Further, future research must refocus the concern from (1) the aquatic to the terrestrial environments, this is where plastic ends up in highest predicted amounts, and (2) testing micro-plastics to testing nano-plastic, as it is the latter that the cells take up.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116363.

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