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Potential for high toxicity of polystyrene nanoplastics to the European *Daphnia longispina*

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Abstract

Background Current regulatory discussions about microplastics are often questioned based on a lack of data indicating high ecotoxic hazards of these particles within standard and recognized definitions. Moreover, there is scientific debate on what metrics to report the micro-nanoplastics toxicity (i.e. mass or particle counts-based exposure). We present here the high potential sensitivity of three genotypically different clones of the European *Daphnia longispina* species complex exposed to non-functionalized polystyrene nanobeads of 50 nm and 100 nm in diameter according to adapted OECD 202 test protocol.

Results $EC_{50}s_{\ 48\ h}$ varied from 0.2 to 8.9 mg L^{-1} (mean 2.49 mg L^{-1}) for 50 nm beads, and from 32.7 to 90.3 mg L^{-1} (mean 59.39 mg L^{-1}) for the 100 nm. $EC_{10}s_{\ 48\ h}$ varied from 0.0007 to 7.5 mg L^{-1} (mean 0.28 mg L^{-1}) for 50 nm beads, and from 25.5 to 69.1 mg L^{-1} (mean 47.51 mg L^{-1}) for the 100 nm. Inter-clonal variability was about tenfold. Therefore, several 1000 s-fold variations in mass-based ecotoxicity for these polystyrene beads was observed if particle size and *Daphnia* genotype are considered jointly.

Conclusions Such ecotoxicity potential is comparable to highly toxic chemicals in global and EU-based regulatory classification and labelling. Ecotoxicity based on particle counts suggested convergence of EC50s, with effects generally observed around 10^{11} to10^{15} particles L^{-1}. The present results highlight the potential high hazard of these particles and the relevance of particle size and exposure metrics on hazard conclusion.

Keywords Genotype, Invertebrate EC50, Dose–response, Polystyrene, Microplastics

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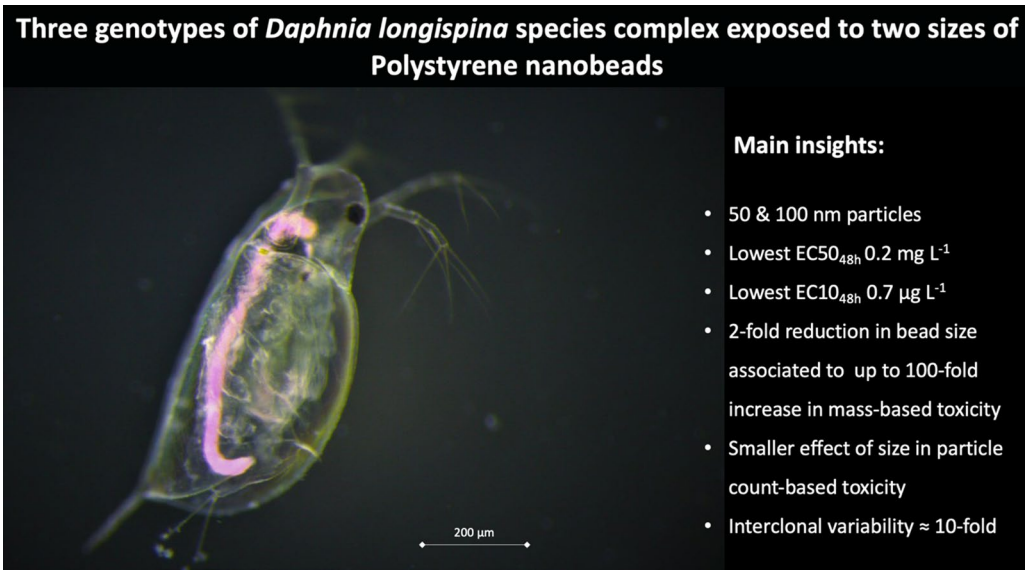
Graphical Abstract



Background

Microplastics (< 5 mm) are suggested emerging threat to marine [11], freshwater [17, 35] and terrestrial organisms [8, 35]. This concern triggered discussions on the need for regulation of the use of these anthropogenic particles. Nevertheless, standard ecotoxicological data supporting high hazard conclusions is not abundant for the smallest fraction of plastic particles–nanoplastics. The precise definition of nanoplastics is under debate, 1000 nm size cut-off was suggested [15]. Such size limit not compatible with the more general definition of nanomaterial or nano-objects under ISO's ISO/TR 80004:2015 (nanomaterial having any external dimension in approximately 1–100 nm length range) or EU regulatory definition of nanomaterial in Recommendation 2011/696/EU *(DG Environment)*. Therefore, a 100 nm cut-off is employed here to distinguish between microplastics and nanoplastics.

Nanoplastics are distinguished from microplastics not only related to their size, but substantial changes in many other properties also follow the decrease in size [8], which affect their potential effects on biota [12]. Conflicting results on hazard across various authors raised questions on the ecological accuracy of toxicity endpoints. The fundamental ecotoxicological relevance and precision of the traditional exposure metrics (particle number counts or mass-based effect concentrations) when assessing the hazard have not been resolved [9]. Some studies report toxicity in mass-based [25] others in particle number-based exposure [11, 17, 26]. Therefore, lack of hazard data and questions on the exposure metrics and intra-species variability contribute to criticism [6] on proposed regulatory action by authorities.

Most of the available evidence regarding the ecotoxicity of micro- and nanoplastics suggest low environmental hazard potential within regulatory definitions (i.e. NOECs and EC_{50}s above 1–10 mg L^{-1}). Values of effect concentrations to 50% of the population (EC_{50}s) for micro- and nanoplastics on mortality and immobilisation in keystone species cladoceran waterflea *Daphnia magna* reported in the literature are variable [38]. Recent hierarchical probabilistic assessments suggest that a threshold of predicted no observed effect in freshwater would be around 0.166 mg L^{-1} [38] and 0.1 mg L^{-1} [42]. These values place plastic particles within materials with arguably low hazard potential, which could change if EC_{50}s lower than 1 mg L^{-1} are reported.

In the present study, we exposed three different morpho-physiological *Daphnia* genotypes to two sizes of polystyrene beads of 100 nm and 50 nm of nominal diameter. These particles are respectively at or below the limit commonly established between microplastics and nanoplastics (i.e. ≤ 100 nm, or ≤ 1000 nm, see above). The model organism is *Daphnia longispina* species complex, one of the most common zooplankton members of permanent water bodies in the northern hemisphere [19, 21, 30]. In majority of previous micro- and nanoplastics studies on the genus *Daphnia*, the standard ecotoxicological model

species was employed—*Daphnia magna* [3]. In contrast to lake species *D. longispina*, *D. magna* inhabits small and fishless habitats and, as a relatively large species, is generally less sensitive to contaminants than small-bodied species, such as *D. longispina* [13, 33]. Thus, the present findings shed light into the hazard properties of small polystyrene beads to this keystone species to lake ecosystems (being main grazer of phytoplankton and main food item for fish). Moreover, by testing multiple genotypes, we provide insights into the potential precision of ecotoxicological metrics performed on single genotypes of tested species.

Methods

Nanoplastic particles

The nanoplastic particles used were polystyrene nanospheres of 100 nm and 50 nm in nominal diameter (hereafter referred as "100" and "50"). These nanobeads were labelled with red fluorescein isothiocyanate dye (Micromod Partikeltechnologie GmbH; product code: 30-00-501; product name: water suspension micromer®-redF). For detailed characterization of 100 nm particles, in terms of their morphology, hydrodynamic size, and electro-kinetic potential, see Schampera et al. [35].

Test organisms

Three *Daphnia* genotypes (hereafter referred to as "clones") of *Daphnia longispina* species complex were used in this experiment: Amme_12, Amme_3, and Amme_51, selected from a wider collection of clones isolated from a natural lake in Germany (Ammersee, 11°10′E, 48°N), in 2008. These clones belong to *D. galeata*×*longispina* F1-hybrids and were previously tested in various experimental surveys [14, 22–24]. The clones have been raised in a laboratory in synthetic medium [33] at 20 ± 2C, 16–8 light–dark photoperiod, and fed three times a week with green algae (*Acutodesmus obliquus*). *Daphnia* were age-synchronized and maintained for two generations under following conditions: five adult *Daphnia* per glass jar containing 200 mL of synthetic medium, fed daily with > 1 mg L^{-1} C of *A. obliquus* (30–40 jars were set up per clone). Neonates of the second-fourth clutch (born within < 24 h) were kept in their original media for 5 days (while all adult *Daphnia* were removed) and these *Daphnia* were then used to start the experiment with.

Experimental design

The range finding experiment was performed with an adult mix of the three clones using five exposure concentrations between 0.001 and 500 mg L^{-1} and a control group, following protocol from OECD 202 [28]. From this range finding it was determined that adult EC_{50} 48 h for this *Daphnia* was generally below 100 mg L^{-1}; and probably contained between 0.1 and 100 mg L^{-1} for exposures of ≤ 96 h. Therefore, seven concentrations per particle size for the current experiment: 0.01, 0.10, 1.00, 2.00, 10.00, 20.00 and 100.00 mg L^{-1}, plus the control (no nanoplastics added) were considered for juvenile exposures. Exposure media consisted of 25 mL of culture media at the appropriate nominal nanoplastic concentration prepared 48 h before the experiment to allow chemical equilibrium between the media ions and nanoplastic surfaces [29].

The 5 day-old juvenile *Daphnia* of each of the three different clones were exposed separately to either one of the two sizes of nanoplastics, at seven exposure concentrations plus control without nanoplastic addition, in five replicates; resulting in 225 experimental units. Each experimental unit consisted of 5 individual *Daphnia*. We used juveniles and not neonates (as otherwise suggested in the OECD guidelines) because the guidelines were developed for *D. magna*, which is a bigger and generally more robust species. Using the neonates for setting up this experiment could have resulted in some random loss of replicates due to handling error.

Assessment of nanoplastic exposure concentration

The nominal exposure concentrations were confirmed using fluorescence measured in extra vials without *Daphnia* (5 replicates per test concentration), at the beginning ($T_{0\,h}$) and at the end of the experiment ($T_{96\,h}$) (quantification limit = 1 mg L^{-1}). From each vial, 1.5 mL was transferred to an Eppendorf tube at $T_{0\,h}$ and $T_{96\,h}$, and stored in the fridge at 4 °C until measurements. The fluorescence emission intensity was assessed using a Tecan 340 fluorescence multi-well plate reader, excitation 552 nm and emission 580 nm. At beginning of the exposure ($T_{0\,h}$, Fig. 1A) there was an exponential increase of the fluorescence related to increase of nominal levels. After 96 h exposure fluorescence in the suspended media dropped significantly proportional to the exposure concentration ($p = 0.0094$), to c.a. 17% and 56% of $T_{0\,h}$ for 100 nm and 50 nm, respectively (Fig. 1B). Given the change in exposure concentrations, only nominal concentrations are referred hereafter. It would not be scientifically defensible to conclude that aggregation and removal from water column yielded the nanoplastics not bioavailable, as we could clearly observe *Daphnia* interacting with them on the bottom of the vials. Nevertheless, the nominal-based EC values are conservative and probably underestimate toxicity given the potential decrease exposure concentration over time.

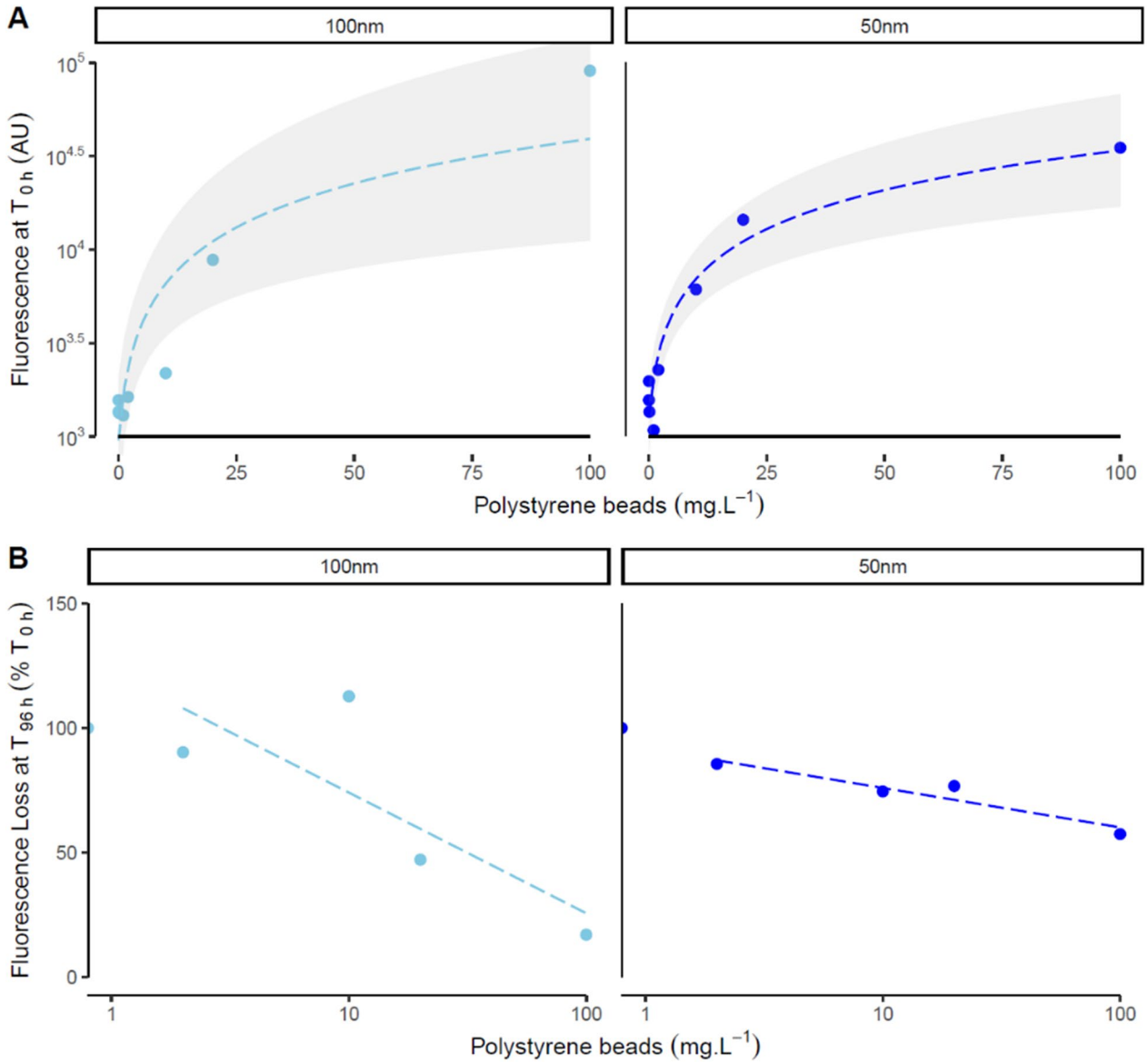


Fig. 1 Relationship between suspended fluorescence and mass of polystyrene particles in exposure media. **A**: The increase in fluorescence for both particles was similar to standard dilution at $T_{0\,h}$. **B:** After 96 h of experiment the fluorescence in suspension at exposure media at 100 mg L^{-1} dropped to c.a. 17% and 56% of $T_{0\,h}$ for 100 nm and 50 nm, respectively. Points represent average of 5 replicates, dashed lines represent regression lines, and in 1A shadow area represents standard error of regression

Experimental procedures

Test *Daphnia* were transferred to the 225 experimental vials with glass pipettes. Experimental units consisted of 5 individual *Daphnia* and all additional procedures followed OECD 202 guideline [28]. Experimental vials were assigned random numbers and relabelled to ensure blind assessment. The number of the immobilized *Daphnia* in each vial was recorded every 24 h during 96 h; at each check dead *Daphnia* were removed. Mortality is not a standard endpoint as per OECD 202 and therefore mortality counts are reported only in Additional file and materials (found at www.abelmachado.com). No food was added throughout the experiment, which was terminated after 96 h. Controls had an average < 10% immobilization, meeting the test validity criteria of 20% (Fig. 2).

Data analysis

All analyses were performed in R [39], and the scripts for computation of statistics, specific data handling details,

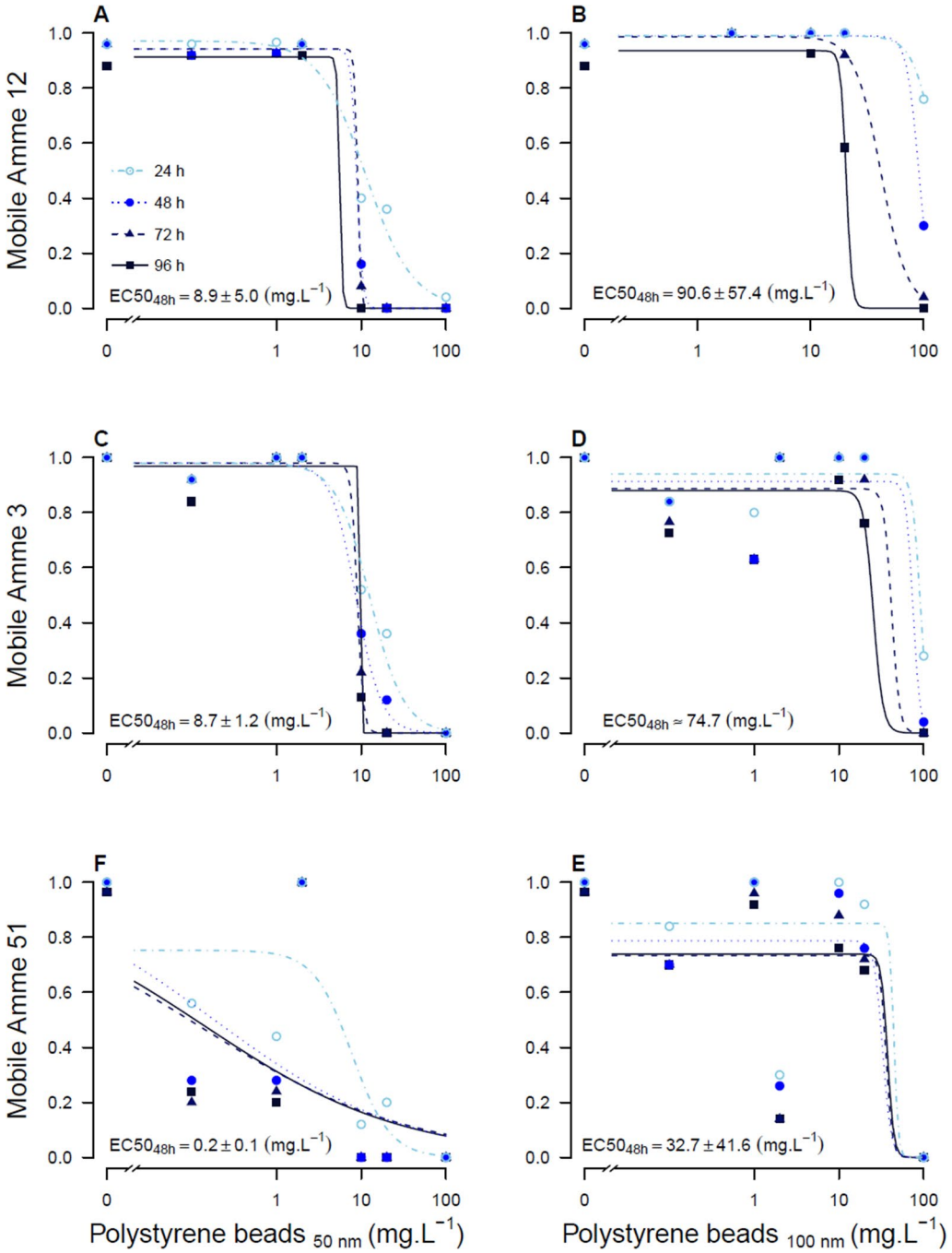


Fig. 2 Effects of plain polystyrene beads on the mobility of three clonal morpho-physiological types of *Daphnia longispina* species complex. Panels **A**, **C**, and **F** refer to dose–response curves after exposure from 24 to 96 h to 50 nm beads whereas panels **B**, **D**, and **E** display data for exposure to 100 nm beads during the same period. Within each panel, light blue unfilled circles represent measured points at 24 h, whereas light blue dotted-dashed lines represent 24 h regression. Likewise, the dark blue filled circles (with dotted lines), black triangle (with dashed lines), and black squares (with continuous lines) represent measured values and regression lines for respectively 48 h, 72 h, and 96 h

and plotting all figures from this manuscript are available in the supporting information linked to this article in the private website of the first author (www.abelmachado.com). Dose-responses were computed using drc library [37] with the drm function for LL3 modelling mobility as binomially-distributed response as function of concentration and time. The related EC_{50}s and confidence interval were obtained with ED function (interval = delta). The

traditional EC_{50} (i.e. mass-based in mg L^{-1}) were converted to other particle metrics, i.e. plastic volume and particle numbers. We computed the total volume of plastic based on the mass and PS density. This total volume was then divided by the volume of a single particle, to obtain an estimation of particle numbers, which was used to obtain total area of particle exposure.

The relationship of computed EC_{50}s over time for the three clones and two particle sizes were modelled with the "*lm*" function (Team, 2022) following formula below:

$$EC_{50} = 0 + I\left(\frac{1}{time}\right) + I\left(\frac{time-1}{time}\right) * Clone * Particle\ Size$$

This equation has the implicit assumptions that (i) there is an inherent relationship between EC_{50} and time, (ii) part of the relationship can be explained by clone and particle size. Graphs we plotted with base *R* + drc plots or with libraries ggplot [40] and cowplot [41].

Results

All three *Daphnia* clones were significantly affected by the two polystyrene particles tested here. The clones Amme_12 and Amme_3 presented similar sensitivity to nanoplastic exposure (Fig. 2A–D). The clone Amme_51 presented c.a. 40-fold higher sensitivity for the 50 nm polystyrene nanobeads compared to other clones (Fig. 2E). For the particles with 100 nm Amme_51 displayed a ~ 2.3-fold increase in toxicity (Fig. 2F). This clone seemed to present some non-monotonicity in the response, with less optimal fit of LL3 models compared to the other two counterparts.

As EC_{10} are the values often used in risk assessments in regulatory contexts, these values are presented in Table 1. Geometric mean is the metric used to compute species specific average sensitivity when multiple data points are available. Geometric means for toxicity of beads of 50 and 100 nm yield values respectively of 2.49 and 59.39 mg L^{-1} for $EC_{50}s_{\ 48\ h}$ and 0.28 and 47.51 mg $L^{-1}$for $EC_{10}s_{\ 48\ h}$.

Generally, effects were observed when particle counts were above 10^{15} L^{-1}. The dynamics of EC_{50}s during the 96 h-exposure is presented in Fig. 3.

Discussion

The current study demonstrates the potential acute toxicity of non-functionalized nano- polystyrene beads to three different clones of *Daphnia longispina* species complex. If ecotoxicity global harmonized hazard identification system (GHS) or classification and labelling of products (CLP) would be applied to these particles, the 50 nm beads would qualify to very toxic to the aquatic environment (GHS and CLP acute ecotoxicity class 1, EC_{50} below 1 mg L^{-1}), while the 100 nm beads would be considered less toxic (GHS acute aquatic toxic class 2, EC_{10} between < 10 mg L^{-1}). However, it is worth to mention that GHS [27], CLP [7], and OECD Tests were designed to address intrinsic toxicity of dissolved chemicals. Later adaptations of those protocols for testing of nanoparticles can be conservative for hazard identification and are to be methodologically considered and interpreted on a case-by-case [29]. When performing tests with nanoplastic suspensions one should also consider that the potential additives contained in commercial dispersions of polystyrene can be an additional cause of toxicity (Brehm et al. [2], Heinlaan et al. [16]). We believe to have minimized this during our experiments as confirmation by the supplier was provided that the product was a water suspension free of any preservatives, and major component left on the suspension was expected to be SO_4 used during final manufacturing steps. A non-target analysis of potential unintended compounds was beyond the scope of this study, however. In fact, the effect concentrations reported here may be underestimated compared

Table 1 Effect concentrations to the 10th percentile after 48 h- exposure considering nanoplastic in terms of mass, particle number, or particle surface. Values presented are average and standard error of estimate

Clone	EC_{10} Mass-based (mg L^{-1})		EC_{10} particle number (count. L^{-1})		EC_{10} particle surface (area nm^2 L^{-1})	
	50 nm	100 nm	50 nm	100 nm	50 nm	100 nm
Amme_12	7.48 ± 10.07^{a}	69.13 ± 164.19^{b}	$2.7e^{15} \pm e^{15a}$	$6.3e^{15} \pm e^{16b}$	$3.5e^{11} \pm e^{11a}$	$2.0e^{11} \pm e^{11b}$
Amme_3	4.09 ± 1.08^{c}	$60.779 \pm na^{c}$	$1.5e^{15} \pm e^{14c}$	$5.5e^{15} \pm na^{c}$	$1.9e^{11} \pm e^{10c}$	$1.8e^{11} \pm na^{c}$
Amme_51	0.0007 ± 0.001^{c}	25.52 ± 25.46^{c}	$2.4e^{11} \pm e^{11c}$	$2.3e^{15} \pm e^{15c}$	$3.1e^{7} \pm e^{7c}$	$7.4e^{10} \pm e^{10c}$

[a] Numbers extracted from model with *p* value = 0.2995

[b] Numbers extracted from model with *p* value = 0.0136

[c] Numbers extracted from model with *p* value < 0.0001

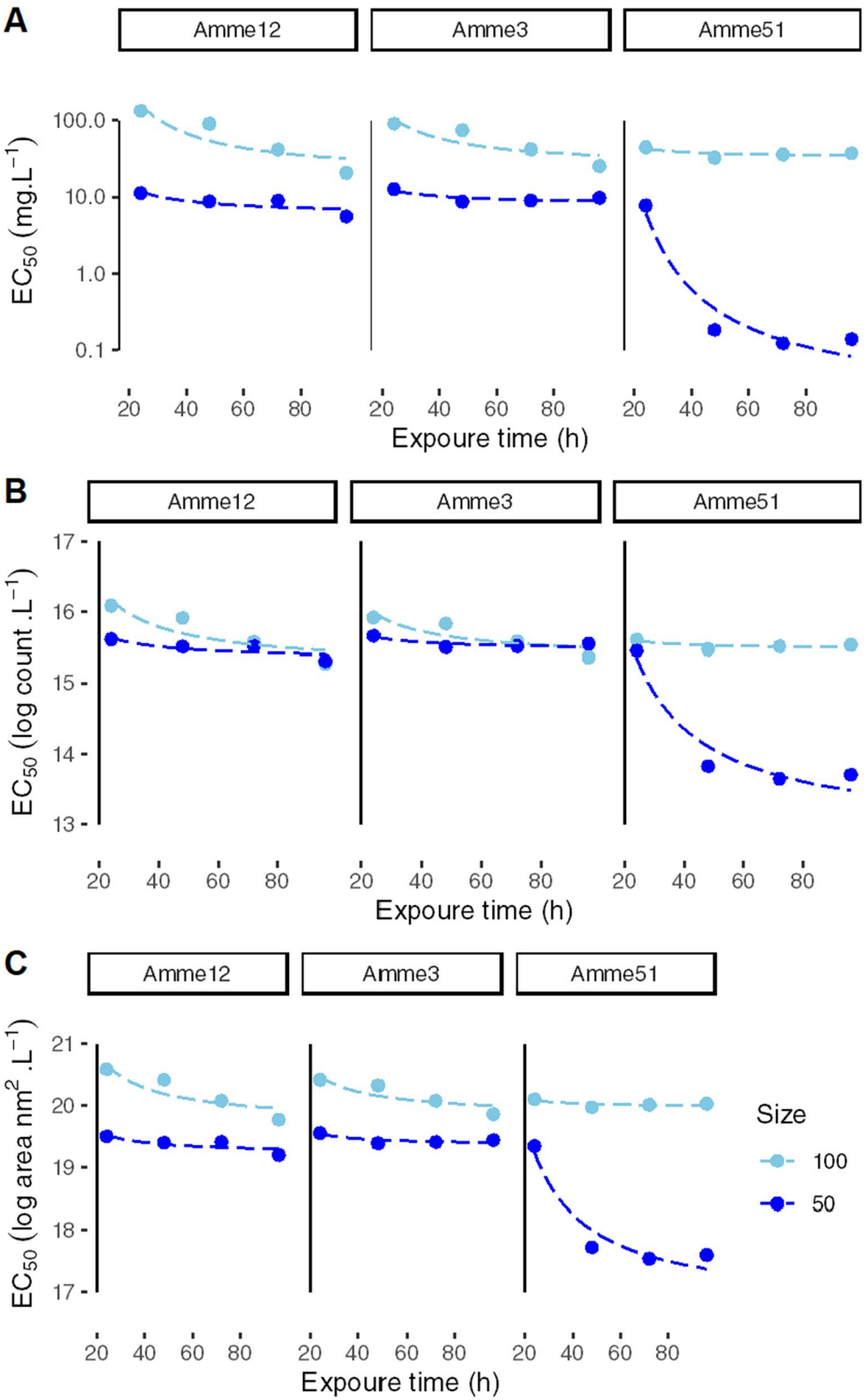


Fig. 3 Temporal dynamics of effects concentration to 50th percentile of *Daphnia longispina* species complex morpho-physiologically distinct clones. Panels display EC_{50}s based on polystyrene beads mass **A**, particle numbers **B**, and particle area **C**. EC_{50} presented here were extracted from dose–response (see Table 1 for statistical significance)

to actual exposure likely being lower than nominal concentration (Fig. 1). There is some evidence that fluorescent-labelled nanoplastics can leach their fluorophores (Catarino et al. [4]) also adding to toxicity, which cannot be fully excluded in this study. Notwithstanding, the current results place 50 nm polystyrene beads amongst the most acutely ecotoxicity category of materials in global and European chemicals regulatory frameworks.

Those particles were not dissolved and it is unclear whether the observed effects could be attributable to intrinsic ecotoxicity in a traditional sense. Thus, the next paragraphs explore three main insights from this experiment under the lenses of elementary ecotoxicological principles.

Variability across *Daphnia* clones

The sensitivity across clones varied c.a. tenfold or more between Amme_51 and Amme_3 within a given exposure-time. Such intra-specific variability in sensitivity is compatible with previous studies that have investigated (dissolved) chemical ecotoxicity and inter-clonal variation in *D. magna*. For instance, Barata et al. [1] found that differences in cadmium tolerance among *D. magna* clones within populations were up to tenfold, while variations among genetically distinct populations in nature could be even greater. Also in previous experiments we observed clonal differences for the tested here set of Ammersee clones; for example, clone Amme_51 was more susceptible to parasitic infection than clone Amme_12 [22], which is consistent with the observed here higher sensitivity of this Amme_51 clone to the nanoplastic exposure.

Intraspecies variability of ecotoxicological responses within *Daphnia* after nanoplastic exposure has not been investigated thus far. However, three studies included multiple *Daphnia* clones in their assessments of toxicity of larger plastic particles (microplastics), showing that these responses are genetically variable [5, 18], even within a single population [32]. Specifically, there was a high inter-clonal variation across the exposed multiple *D. magna* clones: in gene and protein expression [18], in fecundity and growth rates [5] and in life-history and immune responses [32]. There is conclusive evidence of the genetic distance amongst the morphologically and eco-physiological diverse *D. longispina* species complex clones studied here (i.e. Amme_12, Amme_3, Amme_51) [14]. Thus, genetic variation in responses to plastic particle exposure helps to explain some of the contradictory results among *Daphnia* studies based on single genotypes reviewed in [34]). This clone-specific nature of *Daphnia* responses to nanoplastics demonstrates that there might be potential for evolutionary selection and adaptations of populations in contaminated sites. These results strongly support incorporating genetic variation into assessments of the impact of plastic particle exposure.

A twofold decrease in particle size associated to up to 100 s-fold increase in toxicity

The decrease in size of plastic particles can be accompanied by increase in reactivity and occurrence of chemical-like effects [8]. In the present study, the decrease in particle diameter from 100 to 50 nm represented an approximately ~ 100-fold increase in toxicity for the clone Amme_51, and a typical ~ tenfold increase in toxicity for Amme_3 and Amme_12 in EC_{50}s. In fact, reported differences across particle sizes and clones in EC_{50}s are about more than 450-fold [18, 31, 36, 38]. Kögel et al. reviewed that the evidence points towards the tendency of more negative impact by smaller plastic particles compared to larger ones, from the nm to triple-digit µm size range [20]. Adjusting the exposure amount for the different conditions of the size comparisons by mass by most of the existing studies, leads to three orders of magnitude higher particle numbers for each order of magnitude smaller particle diameter [20].

The higher ecotoxicity of smaller particles reported here may be jointly explained by a (i) higher bioavailability and a (ii) higher particle toxicity. The first because 100 nm beads were ~ threefold more prone than 50 nm beads to precipitate out of solution (Fig. 1), yet 100 nm beads were bioavailable (Fig. 4), which is demonstrated by high ingestion rates observed elsewhere [31]. The mild difference in bioavailability of different particle sizes is

Fig. 4 *Daphnia longispina* 48 h-exposed to red-fluorescent dyed 100 nm polystyrene beads at 10 mg L^{-1} quickly displayed digestive tracks full of the nanobeads as evidence of biophysical bioavailability

unlikely to explain alone the much larger differences in sensitivity between the two particle sizes. Moreover, the shape and slopes of dose–response curves observed here within and across exposure times confirms higher toxicity to smaller particles. The observed ecotoxicity of smaller polystyrene beads was seven orders of magnitude higher if EC_{10}s are used as toxicity metric, which suggests a disproportionally stronger potential for chronic ecotoxicity of smaller plastics.

Altogether, the current results support than several 1000 s-fold difference on sensitivity to a particles of a same polymer matrix and chemistry can be expected across studies with the same species when intra-clonal and particle size variability are not addressed.

Considering exposure to plastic mass or particle number

The patterns above-discussed are evident if the exposure metric to determine effect concentrations is mass-based, which is the default metric for hazard identification of materials in ecotoxicology [27, 28]. Nevertheless, most environmental reports of microplastic contamination are based on particle numbers (counts) [10, 17]. Therefore, there are questions on whether exposure to plastic mass, particle number, or other metrics of exposure are ecotoxicological relevant variables for the assessment of micro-nanoplastic effects.

If exposure of *Daphnia* is measured in terms of particle numbers it cannot be consistently asserted whether 100 nm particles are indeed less toxic than 50 nm for all clones (Fig. 3B). In fact, for clones Amme_12 and Amme_3 no significant difference could be observed between 50 nm or 100 nm beads. This suggests similar potency of these individual particles in triggering toxicity for both clones, with $\approx 10^{15}$ particles L^{-1} as the levels for ecotoxicological effects. Nevertheless, less particles of 50 nm beads were required to trigger similar effects on Amme_51 comparted to the 100 nm beads. Despite significant, at the same scale, the difference in Amme_51 sensitivity to the two particle sizes considering particle number-based effect concentration (10^4- fold) is smaller than the difference considering mass-based effect concentrations (10^6- fold). That suggests that normalization of exposure by particle number provides some explanatory power of the particle toxicity, but mass-based inference is still relevant. Such results are coherent to the review of Kögel et al., which did not conclude whether the size or the sheer particle number has the largest impact on the effect [20].

Particles with the same shape were investigated here. Therefore, limited insights can be obtained for some other potentially relevant metrics for micro-nanoparticle as they are redundant to $EC_{mass\text{-}based}$ or $EC_{particle\ number\text{-}based}$. For instance, particle volume is linearly related to particle mass. Similarly, particle surface area co-varies with the product of particle mass, diameter and particle numbers. Further studies with particles of varying shape would be needed to elucidate the relationship amongst these exposure metrics.

Thus, the current results provide evidence that there may be a physical-like component of the micro-nano particle toxicity that can be explained by normalizing exposure values by counts of single particle numbers.

Conclusions

In summary, we demonstrated a high potential acute toxicity of nano- polystyrene beads to three different clones of *D. longispina* species complex that could comparatively place the 50 nm beads amongst the most acutely ecotoxic regulatory classification of materials. We also provided further relevant considerations were made from a fundamental ecotoxicological perspective. The intra-specific variability observed here for the same particle was about tenfold, which is the range observed in intrinsic ecotoxicity assays. However, such difference in toxicity is about 1000 s-fold when particle size and eco-physiologically different clones are jointly considered. Smaller particles were more toxic in this study (i.e., constant shape and chemical composition). Exposure considering particle numbers were relevant descriptors of potentially physical effects; however, mass-based effect concentrations provided additional insights.

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Author contributions

AASM Performed tests, analyzed & interpreted data, wrote the manuscript; NG performed tests, analyzed data, reviewed the manuscript; JW Performed tests, interpreted data, reviewed the manuscript.

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Availability of data and materials

All the experimental results discussed here are freely available as supporting information or upon reauest in the website of www.abelmachado.com. Interested readers may download the data (csv) as well as R commands used for statistical analysis.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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