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# Action of Surfactants in Driving Ecotoxicity of Microplastic-Nano Metal Oxides Mixtures: A Case Study on *Daphnia magna* under Different Nutritional Conditions

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## Abstract

The series of experiments presented in the paper served to clarify the effects of contemporary exposure to surfactant, microplastics (polyethylene and polyvinyl chloride), and nanoparticles (TiO<sub>2</sub> and ZnO) on the model organism *Daphnia magna*. Exposure was evaluated with respect to the age of the organisms (“young”, 24 hours old, and “aged” 10 days old specimens), trophic status (feeding or fasting), and the simultaneous presence of a surfactant. All the above-mentioned substances are present in the wastewater coming from various environmental sources from cosmetic products. The experiments were conducted in compliance with the OECD 202:2004 guideline, which is also a reference for ecotoxicity tests required by REACH. The results showed that surfactants enhance effects of toxicity produced by the exposure to the microplastic + nanoparticle mixtures. The influence due to factors such as nutrition (effect in fasting >> feeding conditions) and the age of individuals (effects in older >> younger animals) is essential. Concerning young individuals, exposure to PE-TiO<sub>2</sub> is the most significant in terms of effects produced: it is very significant, especially in the presence of surfactant (both under fasting and feeding conditions). On the contrary, exposure to the PE-Zn mixture shows the minor effects. The comparison with the literature, especially as regards the possibility of interpreting the toxicity trends for the various mixtures with respect to the individual elements that compose them, leads to hypothesize additive effects still to be investigated and confirms the greatest toxicity contribution of TiO<sub>2</sub>.

**Keywords:** metal-oxide nanoparticles, surfactants, fasting and feeding conditions, microplastics, municipal wastewater treatment plants, toxicity tests

## 1. Introduction

Municipal wastewater treatment plants (MWWTP) are renowned hot-spot sources of a wide variety of pollutants from human activities for the aquatic environments [1]. Nutrients [1], surfactants [2], microplastics (MPs; [3]) and nanoparticles

(NPs; [4]) are significant components of mixtures released by MWWTP, coming from different sources, not least that represented by cosmetic products.

NPs are chemicals, between 1 and 100 nm in size [5], both of natural (i.e., humic and fulvic acids, organic acids, fullerenes, and metals) and artificial origin (TiO<sub>2</sub>, ZnO) [6, 7]. Many products of common use (pharmaceutical and personal care products, plastic, rubber, paints, etc) are based on NPs and this contributes to their massive presence in the environment [8].

MPs can derive from industrial pellets used in the manufacture of plastic objects or from consumer products, such as cosmetics, abrasive products and objects containing microbeads and glitters (primary microplastics) or from the fragmentation of larger plastic objects (secondary microplastics) [3, 9–11]. They are pollutant of great environmental concern [12], affecting feeding habits, and reproductive success of many organisms [13]. NPs and MPs enter the trophic chains when eaten by detritivores and filter feeders [14–17].

Even if wastewater treatment processes retain a large fraction of plastic microparticles [18], in sewage the MPs not removed by plants reach the rivers and, ultimately, the sea [19]. For what concerns NPs, discharges of nano-oxides occur due the sorptive removal of organic contaminants from wastewater [20] purification purposes, together with unintentional releases.

Both, MPs, and NPs are found in the mix of substances present in wastewater and water bodies, together with surfactants. Surfactants have direct toxicity on aquatic species [21] but can also vehicle other substances due to the formation of micelles [22] which can affect pollutant sorption/desorption from MPs surfaces [23]. Surfactants could represent the way to increase the interaction among microplastics and animals and therefore lead to negative effects on exposed animals [22].

Toxic effects due to the exposure to complex mixtures differ from the exposure to single substances, even if compounds are at low levels [24]. In this case, NPs and MPs toxicity could be affected both by the nutrient induced microalgal growth and by synergic/antagonistic interactive effects due to surfactants [25]. The presence of metal-oxides NPs, MPs, and surfactants in effluents from MWWTP suggests deepening their ecotoxicity to assess the real effects on aquatic environments.

Despite the increasing interest, recent meta-data analysis underlined low standardization of in vitro tests [25] and the largest number of experiments performed on single kind MP/NP. In Europe, the placing on the market of new formulations implies the verification of their environmental compatibility in accordance with the current regulation (REACH).

*Daphnia magna* (Cladocera) is a crucial model freshwater species for ecotoxicological tests due to the well standardized procedural practice (i.e., OECD standards) for experimental exposure [26].

This study aims to fill some important knowledge gaps on NPs and MPs ecotoxicity. It evaluates ecotoxicological responses of *D. magna* exposed complex mixtures (NPs + MPs in presence/absence of surfactant). Effects attained under fasting conditions (OECD guidelines) are matched to those achieved following the exposure of animals under feeding conditions, supposed as natural and with animals of different ages.

## 2. Material and methods

### 2.1 Experimental design

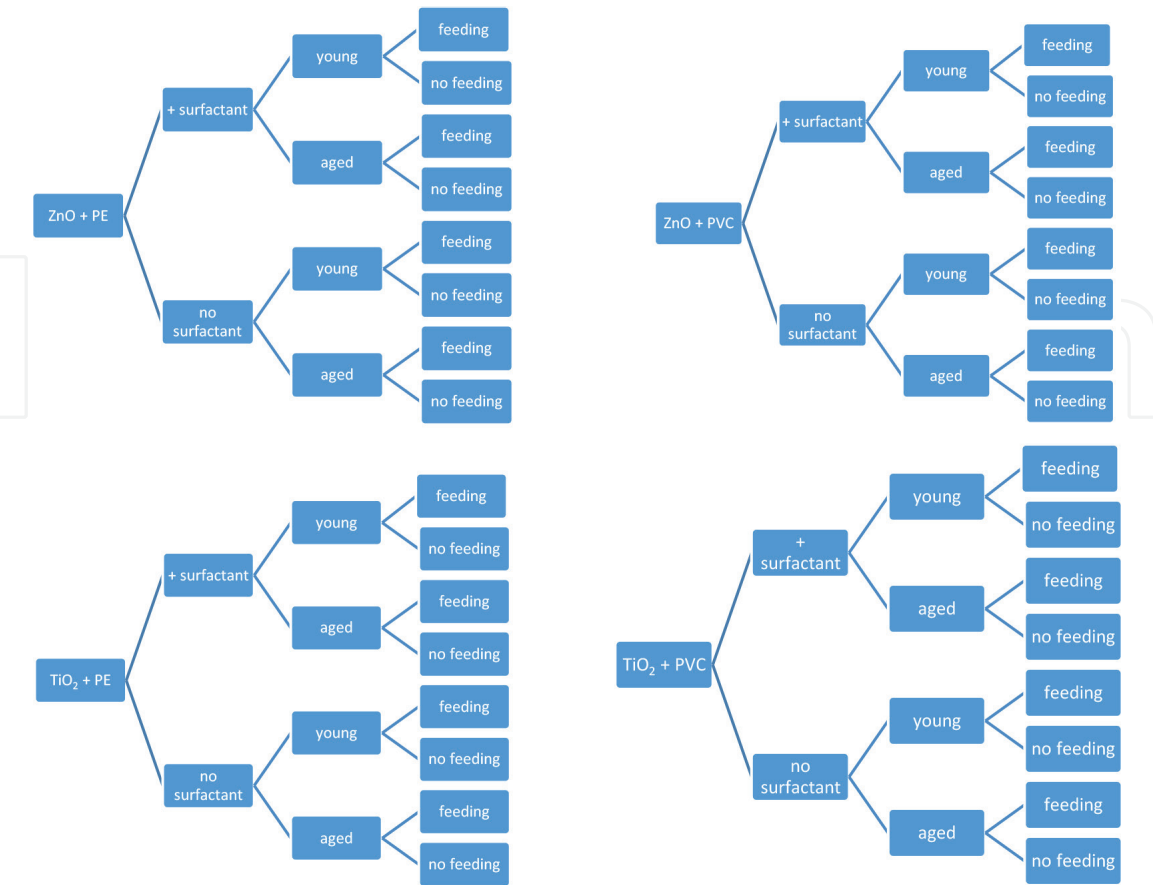
The experiments were performed under the OECD 202:2004 guideline [27]. Dispersions were made by suspension of tested particles in UNI EN ISO 6341:2012 standard freshwater. Experiments previously reported [28, 29] allowed to

determine for each toxicant the dose permitting the survival of a significant fraction of the tested population until the end of the exposure time (96 h).

**Figure 1** summarizes the experimental design. Mixtures of NPs (n-ZnO and n-TiO<sub>2</sub>) and MPs (PE and PVC) were used for the exposure experiments, adding/not adding a non-ionic surfactant (Triton X-100, CAS n. 9002-93-1; tested at 0.001% v/v according to [22]) to improve the dispersion of MPs and NPs in tested samples; results were compared to controls to test ecotoxicological effects of the NPs-MPs and NPs-MPs-surfactant mixtures. Furthermore, we also exposed to dispersions of microplastics + surfactant animals that at the beginning of the experiment had 10 days of life (called “aged”) to evaluate the effect of aging on the ecotoxicological responses. Animals were exposed under fasting conditions, selecting immobilization as endpoint and a contact time 24-48 h. Contextually to standard conditions required by OECD, animals were also exposed under feeding conditions and contact time was extended from 24 to 48 h to 96 h as suggested by the literature for tests performed on particulate toxicant [30] performing observations daily starting from T<sub>24</sub> after the initial exposure. Experiments were made during an 8:16 dark/light exposure cycle [31].

ZnO and n-TiO<sub>2</sub> were tested at 1.12 and 113.18 mg/L respectively; microplastic doses were 0.05 mg/L. The selection of microplastics to be tested was done according to Renzi et al. [29].

Dispersions of both single metal-oxides NPs, MPs and mixtures were characterized by microscopy coupled to Fourier Transformed-Infrared spectrometer (μFT-IR, model Nicolet i-10 MX equipped with ATR detector, Thermo®). The formation of clusters of nanoparticles in the mixtures was verified at the micrometric scale even in conditions with the addition of food. Survival rates % of exposed animals compared to negative controls were used as target endpoints.



**Figure 1.**  
Logic model of the exposure experiments on *D. magna*.



## 2.2 Equipment and materials

Experimental condition for *D. magna* (MicroBioTest Inc. ephippia) storage, hatches and preliminary treatments were the same described by Renzi and Blašković [28]. Collection of organisms was standardized at 90 h after the start of incubation. All reagents were purchased from Caelo or Sigma-Aldrich. Experimental conditions and sets were the same previously described by Renzi et al. [29] and Renzi and Blašković [28] to allow a comparison of reported data on toxicity of mixtures tested in this study with results obtained by previous research on single components of the tested mixtures. Also, experimental conditions are the same to allow a complete comparability of obtained results. Chemicals features and properties of nanoparticles, microplastic, and surfactant tested in this study are the same for chemicals reported by Renzi et al. [29] and Renzi and Blašković [28].

## 2.3 Quality assurance and quality control

Ecotoxicological tests were performed following UNI EN ISO 17025 guidelines to ensure quality control of collected results. Laboratory performed experiments ensured to pass inter-calibration exercises performed on annual basis on *D. magna* immobilization. During experiments pH and DOM were measured to verify that they remained within the limits of acceptability defined by OECD (202:2004) [27]. Mortality of animals in negative controls shall be included within 0-10% not to invalidate tests.  $LC_{50}$  of chemical solution used as reference to test animals' responses was acceptable (0.6–2.1 mg/L). Test were performed in triplicates ( $n=3$ ); results obtained by the exposure to samples were compared statistically with responses obtained by negative controls; significance ( $p<0.01$ ) of observed differences among mean values was tested by T-test while differences among variances were explored by F-test (Prism® 4.0). Results reported in this study are mean values (standard deviation, SD), normalized concerning negative controls.

## 3. Results and discussion

The results obtained from the exposure experiments are summarized in **Table 1** (young specimens) and **Table 2** (aged specimens). In the following text, the term significant refers to statistically significant based on the results of the statistical analysis carried out on the results obtained from the exposure experiments considering immobilization as the endpoint.

As for both young and aged individuals, exposure to PE-TiO<sub>2</sub> is the most significant in terms of effects: it is very significant, especially in the presence of triton (both fasting and feeding). On the contrary, exposure to the PE-Zn mixture shows the minor effects (**Figure 2**). This result, not linear if we consider that there is no clearly identifiable trend in the toxicity levels of the NPs/MPs, leads to think that more than the actual toxicity of the individual elements of the mixtures, there is a significant additivity effect. This estimation would also be legitimized by the comparison with previously obtained results: PVC, in presence of surfactant, resulted the most toxic among tested dispersions for both neonates and aged *Daphnia magna* exposed [29].

On the other hand, in Renzi and Blašković [28], n-ZnO resulted less effective than n-TiO<sub>2</sub> in leading to the target endpoints (immobilization and death) in exposed *Daphnia magna* individuals; in this sense the observations made in this study would confirm a higher toxicity contribution of n-TiO<sub>2</sub> compared to n-ZnO. A further factor to be taken into consideration in the interpretation and future

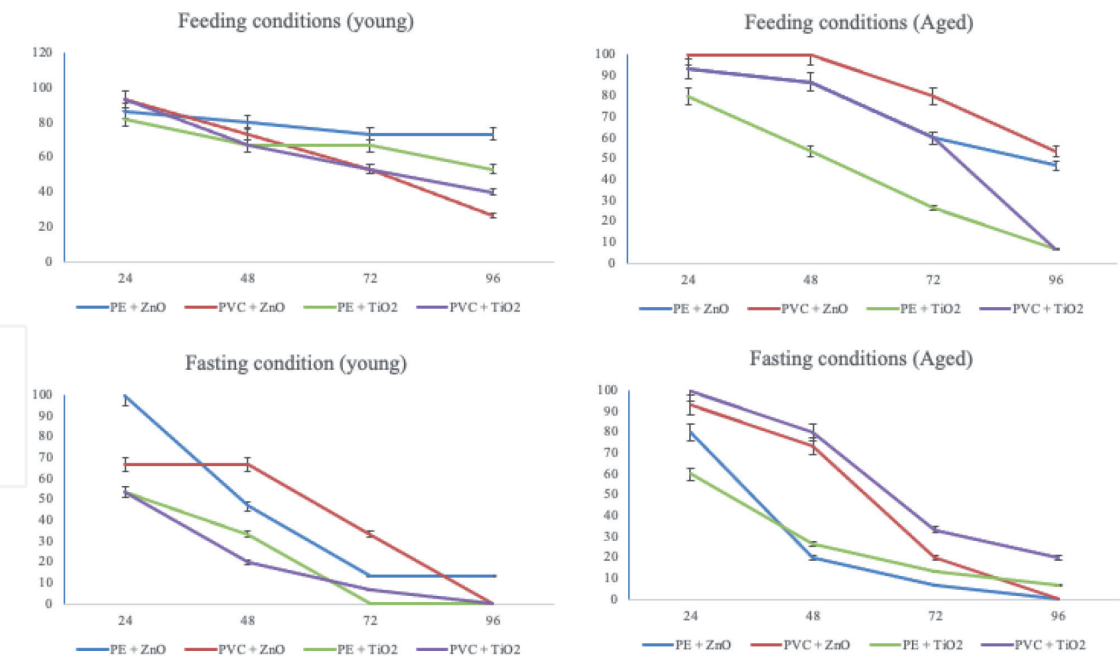
		Feeding conditions (MPs 0.05 mg/L; n-TiO <sub>2</sub> 113.18 mg/L)				Feeding conditions (MPs 0.05 mg/L; n-ZnO 1.12 mg/L)			
Surfactant		no	no	yes	yes	no	no	yes	yes
MPs type		PE	PVC	PE	PVC	PE	PVC	PE	PVC
24 h	%Effect	6.67	6.67	17.78	6.67	0.00	0.00	13.33	6.67
	DS	0.00	0.00	9.62	11.55	0.00	0.00	0.00	11.55
96 h	%Effect	20.00	26.67	46.67	60.00	6.67	33.33	26.67	73.33
	DS	0.00	11.55	11.55	11.55	0.00	11.55	11.55	0.00
		Fasting conditions (MPs 0.05 mg/L; n-TiO <sub>2</sub> 113.18 mg/L)				Fasting conditions (MPs 0.05 mg/L; n-ZnO 1.12 mg/L)			
Surfactant		no	no	yes	yes	no	no	yes	yes
MPs type		PE	PVC	PE	PVC	PE	PVC	PE	PVC
24 h	%Effect	6.67	13.33	46.67	46.67	0.00	86.67	0.00	33.33
	DS	11.55	0.00	20.00	0.00	0.00	11.55	0.00	20.00
96 h	%Effect	53.33	60.00	100.00	93.33	46.67	100.00	80.00	100.00
	DS	11.55	0.00	11.55	11.55	11.55	11.55	0.00	11.55
Surfactant (Triton X-100).									

**Table 1.**  
Percentages observed for the “immobilization” endpoint in young individuals, in relation to experimental exposure parameters.

		Feeding conditions (MPs 0.05 mg/L; n-TiO <sub>2</sub> 113.18 mg/L; Surfactant)			Feeding conditions (MPs 0.05 mg/L; n-ZnO 1.12 mg/L; Surfactant)	
MPs Type		PE	PVC		PE	PVC
24 h	%Effect	20.00	6.67		6.67	0.00
	DS	11.55	11.55		11.55	0.00
96 h	%Effect	93.33	93.33		53.33	46.67
	DS	0.00	11.55		11.55	11.55
		Fasting conditions (MPs 0.05 mg/L; n-TiO <sub>2</sub> 113.18 mg/L; Surfactant)			Fasting conditions (MPs 0.05 mg/L; n-ZnO 1.12 mg/L; Surfactant)	
MPs Type		PE	PVC		PE	PVC
24 h	%Effect	40.00	0.00		20.00	6.67
	DS	20.00	0.00		23.09	11.55
96 h	%Effect	93.33	80.00		100.00	100.00
	DS	11.55	20.00		0.00	0.00
Surfactant (Triton X-100).						

**Table 2.**  
Percentages observed for the “immobilization” endpoint in aged individuals, in relation to experimental exposure parameters.

analysis of the results is the ability to interact at a chemical level between surfactant and MPs, which could be material dependent. This difference between plastics of different nature, already hypothesized [32], could explain the variability of the results obtained in terms of toxicity of different mixtures.

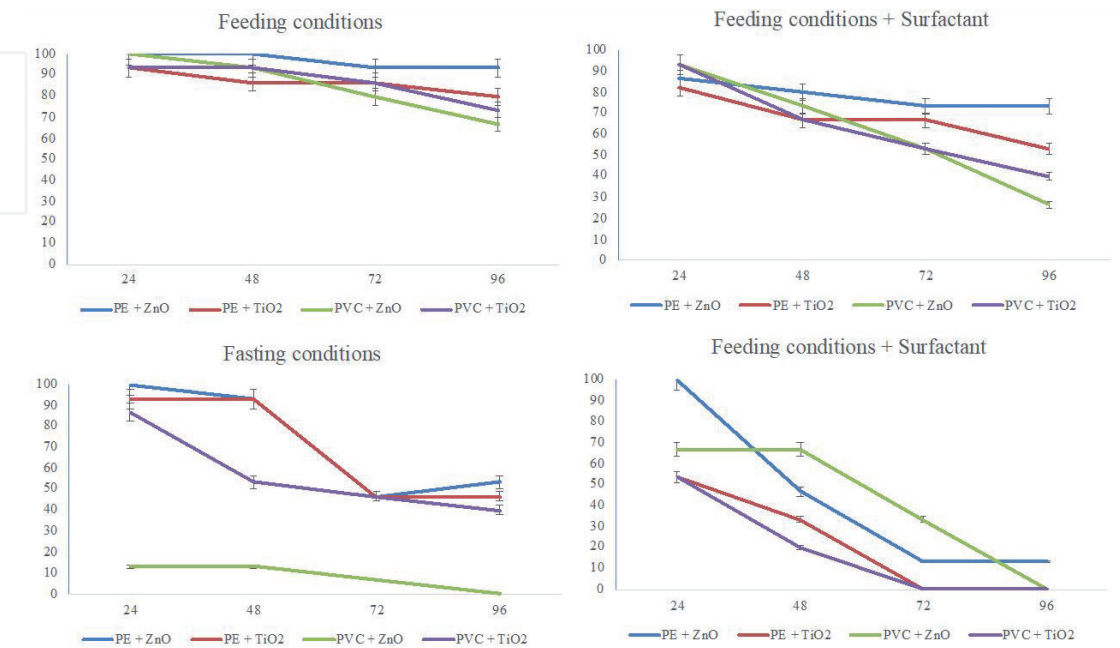


**Figure 2.** Comparison between young and adult organisms exposed in surfactant presence and fasting/feeding. Graphics report the mean percentages of mobile organisms at the target time normalized for negative controls.

### 3.1 Surfactant effect

The effect of surfactant (Triton X-100) was significant in all the experiments carried out on young organisms, compared to the control batches without the exposure to surfactant. For this reason, and to simplify the factors considered, making the observed effects more evident, it was decided to always add surfactant to the mixtures of contaminants to which adult organisms were exposed.

**Figure 3** shows the contribution of surfactant presence/absence under different trophic conditions in young organisms exposed to NPs+MPs mixtures, in terms of percentages of mobile organisms at the target times. These effects could be because



**Figure 3.** Contribution of surfactant presence/absence and trophic condition in young organisms. Graphics report the percentages of mobile organisms at the target time normalized for negative controls.

surfactants seem to improve the contact among microplastics and animals and therefore cause effects on exposed specimens [22]. In a previous study [29], exposure to microplastics + surfactant showed the highest toxicity on *D. magna*, also, supporting results obtained on tested complex mixture.

### 3.2 Feeding effect

The effect of feeding is almost always significant (**Figure 3**) on the toxicity of mixtures of contaminants on *D. magna*. Animals actively ingest nanoparticles during both fast and feeding conditions, but feeding condition reduces the effects of mixtures on the exposed animals. In young organisms, the only case in which the effect of food was not significant was due to the exposure to PE in the presence of n-TiO<sub>2</sub>.

In the case of adult individuals (**Figure 2**), the effect of feeding was significant in 5 out of 8 cases: in each experiment for PE+n-TiO<sub>2</sub>, partially following to exposure to Zn (with PE and PVC always at 96 hours) and with PVC+n-TiO<sub>2</sub> at 24 hours.

It can be hypothesized that with the intake of food (phytoplankton) there is an increase in the metabolic rate, resulting in a better chance of detoxifying by *D. magna*, making the effects of toxic mixtures less evident. Concerning n-ZnO, another reason may be that, as the toxicity of metal-oxide nanoparticles reasonably due to the release of metal ions in water [33] some water chemical parameters, such as increasing pH and DOM (occurring during feeding with phytoplankton) could have reduced the concentration of free Zn<sup>2+</sup> released from n-ZnO. Feeding could have affected these key parameters (DOM and pH in particular) following the addition of an external organic source (DOM) and due to changes induced by photosynthesis performed by algal activity on water chemical features as, also, previously hypothesized for single components of complex mixtures tested in this study [28].

### 3.3 Aging effect

The results achieved provides evidence that the level of toxicity of NPs+MPs mixtures depends on the age of the animals, confirming what yet seen following tests with several kinds of MPs [29]. The effect of aging (comparison between experiments carried out on young people and adults, **Figure 2**) was significant (affecting the possibility of remaining mobile or alive) in half of the experiments carried out. While results at 24, 48 and 72 hours appear controversial, the significance of a toxic effect clearly emerges at 96 hours, both for PE+n-ZnO and for PE+n-TiO<sub>2</sub>. Significant aging effect also for PE+n-TiO<sub>2</sub> at 96 hours and PVC+n-TiO<sub>2</sub> at 96 hours have been registered.

## 4. Conclusions

The results obtained in this study made it possible to identify the mixture PE+n-TiO<sub>2</sub> as the most toxic following exposure of *D. magna*, both in the presence of surfactant and without, both under fasting and feeding conditions, in the adults and young specimens tested. The variability and, in many cases, the lack of a univocal trend in the toxicity effects observed between mixtures of different compositions, is reasonably attributable to additive effects to be studied further. On the other side, data that emerge and substantially confirm observations previously performed by research on exposure to single toxicants are: i) the influence of fasting or feeding conditions on toxicity of tested mixture; feeding specimens suffer less from the effects of exposure to contaminants than others; ii) the presence of surfactant (Triton X-100) increases the toxic effects of tested mixtures of contaminants;



iii) adult specimens showed a different resistance to the effects of exposure to mixtures of contaminants than younger specimens (target time 96 hours). Results obtained in this study, furthermore, highlight as interaction between surfactant and other chemicals/materials could induce ecotoxicological responses that cannot be predicted only based on single component tests. This effect is particularly relevant in the real world when animals are exposed under feeding conditions and for longer exposure time than during standardized tests.

### Acknowledgements

Authors are grateful to BsRC research centre (Italy) that founded and completely supported this research. Funding: This work was supported by Bioscience Research Center [grant number RG2020009].

### Abbreviations

MWWTP	municipal wastewater treatment plants
MP	microplastic
NP	nanoparticle
TiO <sub>2</sub>	titanium dioxide
ZnO	zinc oxide
REACH	European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals
OECD	organization for economic co-operation and development
PE	polyethylene
PVC	polyvinyl chloride
μFT-IR	micro Fourier transform interferometer
ATR	attenuated total reflection
DOM	dissolved organic carbon

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