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Effects of Different Microplastic Types and Surfactant-Microplastic Mixtures Under Fasting and Feeding Conditions: A Case Study on *Daphnia magna*

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Abstract
This study evaluates the mortality and immobilization on *Daphnia magna* after 24–96 h of exposure to microplastic dispersions (PP, PE, PVC, PVC/PE), and to microplastic + surfactant solutions both under fasting and feeding conditions. The tested microplastics were analysed with μFT-IR to determine their chemical composition, purity, and dimensions. The results show that: (i) exposure under fasting conditions produces acceptable results on negative controls no later than 24 h; (ii) the dispersion of microplastics forms homo-agglomerates that are able to affect animals' motility and cause mortality and immobilization; (iii) different types of tested microplastic produce different effects on endpoints (the most toxic is PVC + surfactant); (iv) in all cases, the effects were reduced under feeding conditions (i.e. 4 times reduction of PE toxicity); (v) effects of surfactant on observed toxicity are microplastic-type dependent; (vi) the age of the animal affected the mortality and immobilization responses after exposure under both fasting and feeding conditions.

Keywords Microplastics · *Daphnia magna* · Surfactants · Fasting and feeding conditions · Freshwater environments

Marine litter represents a new pollutant type of great environmental concern (Browne et al. 2007) as indicated in Europe by the "Horizon 2020″ program under the Marine Strategy Framework Directive (2008/56/EC). Plastic represents the principal component of marine litter. Microplastics, in particular, are widespread in water and sediments (Browne et al. 2011; Free et al. 2014; Kim et al. 2017), impacting feeding habits, and reproductive success of many organisms (Cole et al. 2013). The transfer of the particles from the environment towards the aquatic trophic webs which represents an important direct threat affecting ecosystems (Kim et al. 2017), was recorded many times. The principal route of transfer is represented by filter feeders and detritivores (Wright et al. 2013; Renzi et al. 2018a, b). Surfactants are detected frequently at significant concentrations in coastal aquatic environments, due to effluents from municipal wastewater treatment plants (Renzi et al. 2012). Surfactants can show direct toxicity on aquatic species (Lechuga et al. 2016) but can also transport other chemicals due to the formation of micelles (Frydkjær et al. 2017) which can affect pollutant sorption/desorption from microplastics (Bakir et al. 2014). Recent researches evaluated ecotoxicological effects caused by the ingestion of microplastics on aquatic species (Wright et al. 2013; Galloway and Lewis 2016). Surfactants could be the key to improve the contact among microplastics and animals and therefore cause effects on exposed animals (Frydkjær et al. 2017). Nevertheless, the toxicity of different types of microplastics and their interactions with surfactants have not yet been clarified in the literature. *D. magna* (Cladoceran) represents a key model freshwater species for ecotoxicological studies due to the well standardised methodological protocol for laboratory exposure (Baird et al. 1989). This study aims to fill the knowledge gaps reported by the recent literature on effects after 96 h of exposure to different types of irregularly shaped microplastics using *D. magna* as model species. Endpoints of toxicity to *D. magna* were evaluated with and without the presence of surfactants, under fasting and feeding conditions, and with animals of different ages.

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Materials and Methods

Details on the design of the experiments, the treatment of materials and the methods followed for the exposure of animals are reported in Supplementary material. According to the protocols of ISO 6341 (OECD 2004), early stages of *D. magna* were exposed to: (i) different microplastic types (range size of 10–100 µm) as: polyethylene (PE), polyvinylchloride (PVC), polypropylene (PP), Polyvinylchloride/Polyethylene (PVC/PE); (ii) surfactant (S); (iii) microplastic + surfactant mixtures. Exposure tests were performed under both fasting and feeding conditions. Some experimental variables were selected according to results obtained by Frydkjær et al. (2017). These included the toxicity endpoints observed (mortality and immobilization), microplastic doses (0.05 g/L), and microplastic/surfactant percentages (0.001% v/v). Surfactants tested in this study were purchased by Sigma-Aldrich, and associated LC50 for this chemical in *D. magna* is within 18–26 mg/L after 48 h of exposure. We classified animals as immobile if they lost vertical swimming capability after gentle agitation of the liquid for 15 s as reported by ISO 6341:2012(E). The exposure time was 96 h according to Baumann et al. (2014) for the evaluation of ecotoxicological effects following exposure to particulate materials. Tests were performed on irregularly shaped microplastics (Frydkjær et al. 2017). The selection of microplastics to be tested was done under the microscope (Nikon, mod. SNZ-800 N) through a steel sieve (mesh diameter of 100 µm). Nikon's coloured high-resolution video camera (Nikon ACT-1) and real-time image analysis software (Nikon DS-Fi2) enabled repeated measurements of the sample size smaller than 100 µm mesh (further details are reported in Supplementary materials). We chose Triton X-100 as a representative substance of the category of non-ionic surfactants due to its wide use in cleaning products. Moreover, it is a good dispersant of chemical substances (Zhang et al. 2013) and we used it to improve the dispersion of microplastics in tested samples. 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. Before starting the experiments we chemically characterized the microplastics rinsed with pure ethanol to exclude contamination (Supplementary material). Chemical analyses were performed using µFT-IR (Nicolet i-10 MX - ATR model, Thermo®). Data on mortality and immobilization responses (stereomicroscope model SNZ-800 N, Nikon®) were statistically analysed using Prism® v.5.0 (GraphPad Software Inc) to evaluate the significant of the observed differences between treatments and controls ($p < 0.01$).

Results and Discussion

Microplastics produce different mechanical effects on exposed animals (Fig. 1). Algae absorb on irregular surfaces of tested microplastics (Fig. 1a, tests under feeding conditions). The formation of homo-agglomerates of microplastics produces significant effects of entrapment making difficult the swimming activities of the exposed animals (Fig. 1b, c, f).

The presence of surfactant increases the formation of homo-agglomerates and promotes microplastic adhesion on animal appendages. *D. magna* actively ingests the microplastics under both fasting and feeding conditions. Aged animals showing the ingestion of microplastics are reported in Fig. 1e.

In Fig. 2, percentages of mortality (2a) and immobilization (2b) under fasting conditions are compared to controls (green lines). After 24 h of exposure, only PP and PVC + S show mortality (even if the EC20 is not calculable). However, almost all types of tested microplastics show immobilization > 10%, with the exception of PVC / PE (5%), PP (5%) and PVC (0%). Nevertheless, the statistical analysis provides evidence that only surfactant exposure induced a significant effect ($p < 0.01$). After 48 h, under fasting conditions mortality increases significantly in all tested samples and, after 96 h, percentages of mortality are always > 30%. PVC + S dispersion results more toxic than others and of the 20% higher than negative controls. All the other tested dispersions under fasting conditions result less toxic than negative controls. Concerning immobilization, microplastic + S show higher average effects compared to microplastics dispersions alone. As regards immobilization, the presence of surfactant (microplastics + S) increases the toxicity of microplastics. PP and PE are more toxic than PVC, while PVC + S is more toxic than PP + S and PE + S. Data on exposure under feeding conditions are shown in Fig. 3a, b. Mortality is observed starting from 48 h but only the surfactant (S) shows significant difference compared to controls ($p < 0.01$). After 96 h recorded effects are significant for almost all materials. Exposure to microplastics + surfactant shows the highest toxicity (PVC + S > PE + S > PP + S > S > PE; $p < 0.01$). In Table 1, ratios between effects recorded under fasting and feeding conditions are reported to synthetically represent observed trends.

Surfactant increases the mortality and the immobilization in all tested dispersions, both under fasting and feeding conditions (Fig. 2–3; Table 1). After 24 h under fasting conditions (standard OECD tests), surfactant results the most toxic even though EC20 is not calculable. After 48 h, PE + S shows 33.3% of immobilized animals compared to the lower levels recorded by S (27.8%) and PE (26.7%) separately. A similar behavior is recorded under feeding conditions

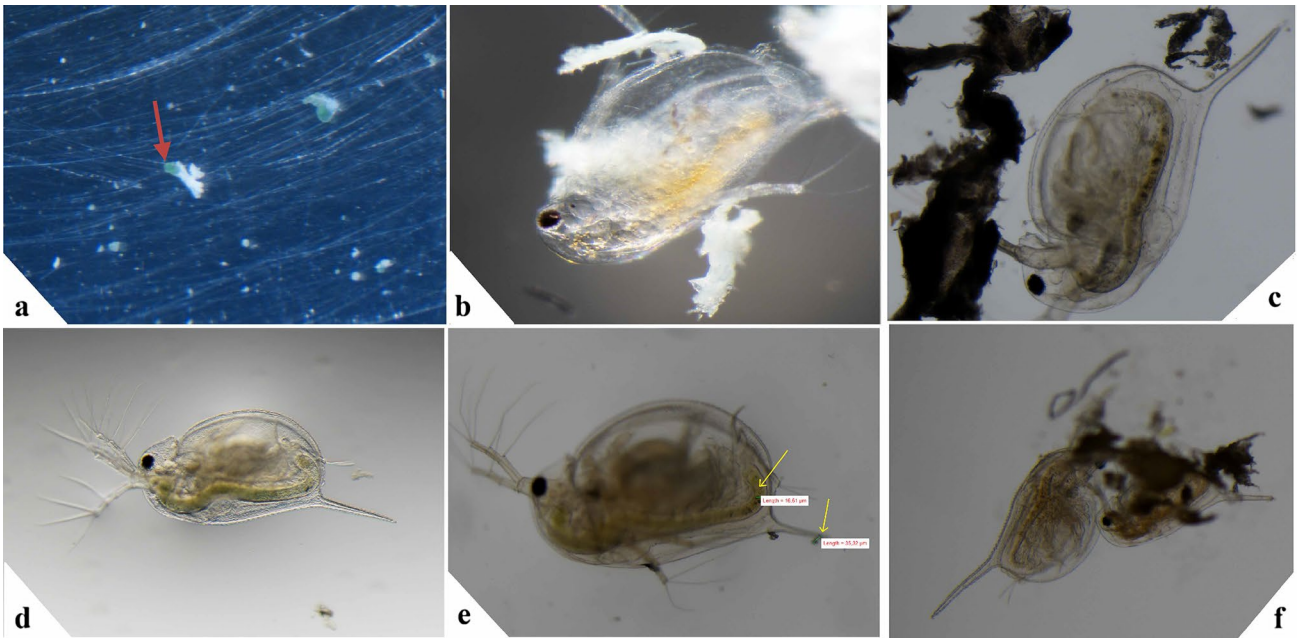

Fig. 1 Microscopic effects. **a** Interactions between Spirulina spp. (algae, used to fed *Daphnia magna*) and PVC microplastics (arrow indicates the superficial coating by algae on plastic surfaces); **b**, **c** interactions between *D. magna* and PVC under fasting (**b**) and feeding conditions (**c**); **d** fed control; **e** Aged *D. magna* with Spirulina and PVC microplastics inside the digestive systems and outside (arrows). Dimensional differences between ingested microplastics and floating microplastics are highlighted; **f** aggregation of *D. magna* trapped into a PVC cluster

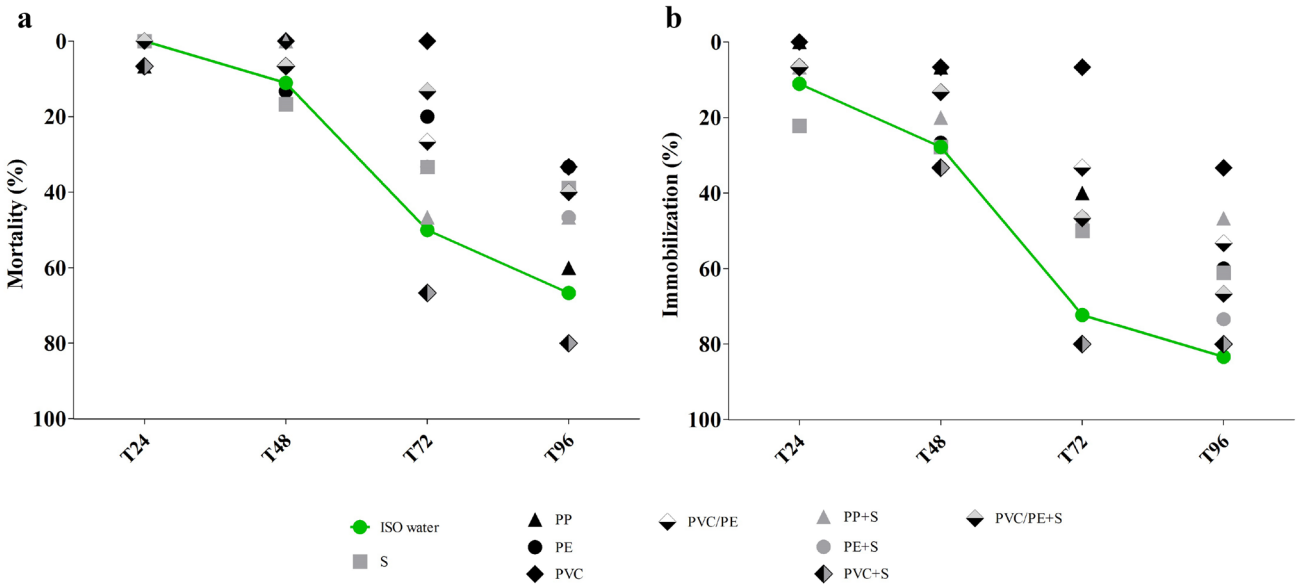

Fig. 2 Average results obtained under fasting conditions. At any experimental time, mortality (%) (**a**) and immobilization percentages (%) (**b**) are reported. Standard deviations were not represented to improve the figure clearness nevertheless, recorded SD ranged within 0%–23.1% in both cases (**a**, **b**). Tested microplastic dispersions doses are fixed (0.05 g/L) as well as surfactant doses (0.001% v/v). Surfactant (S) tested was Triton X-100. Positive controls were tested; results obtained after the exposure to $K_2Cr_2O_7$ for 24 h show animals' responses within OECD (2004) standards (EC50 0.6–2.1 mg/L). Concerning negative controls (green lines), mortality is zero at 24 h and within the OECD (2004) guideline standards on this species. Under fasting conditions mortality rates increased quickly over the time reaching 11.1% ± 19.2% after 48 h and 66.7% ± 0.0% at 96 h. Immobilization in negative controls early reaches 20% (27.8% ± 16.7% after 48 h) and 85.3% at 96 h (25.5% SD)

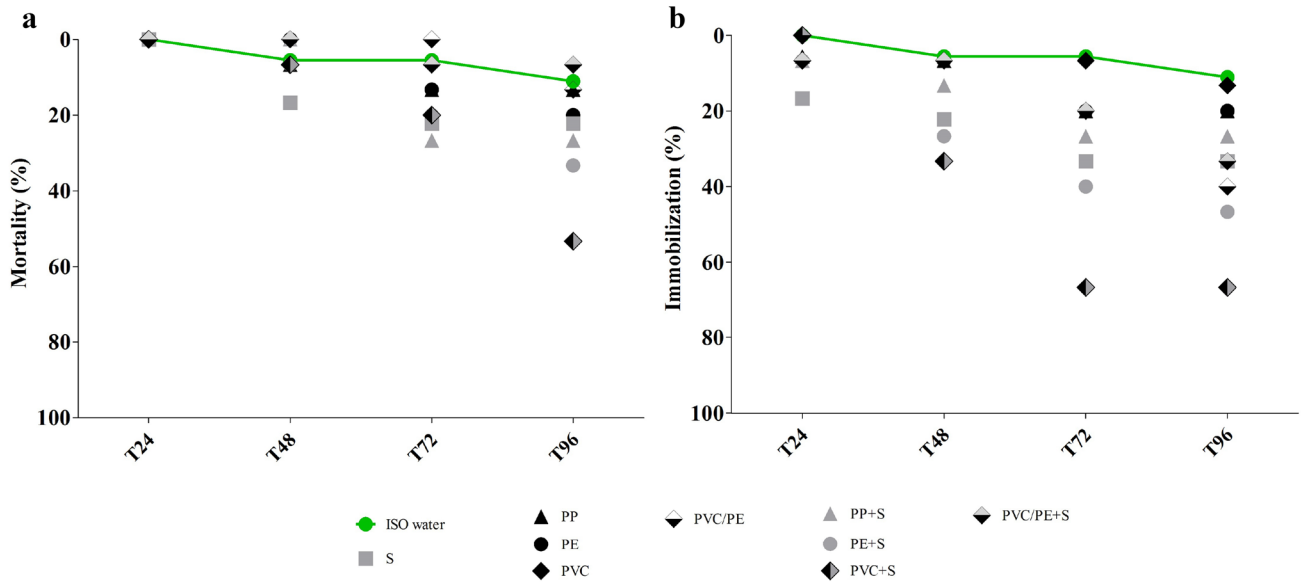

Fig. 3 Average results obtained under feeding conditions. At any experimental time, mortality (%) (**a**) and immobilization percentages (%) (**b**) are reported. Standard deviations were not represented to improve the figure clearness nevertheless, SD ranged within 0%–11.5% (**a**) and 0%–20% (**b**). Tested microplastic dispersions doses are fixed (0.05 g/L) as well as surfactant doses (0.001% v/v). Surfactant (S) tested was Triton X-100. Positive controls were tested; results obtained after the exposure to $K_2Cr_2O_7$ for 24 h show animals' responses within OECD (2004) standards (EC50 0.6–2.1 mg/L). Concerning negative controls (green lines), mortality is zero at 24 h and within the OECD (2004) guideline standards on this species. Under feeding conditions, mortality is constant (5.6% ± 9.6%) within 48–72 h and 11.1% ± 9.6% at 96 h. Immobilized (%) shows the same behaviour described by mortality evidencing low effects also after 96 h

Table 1 Ratio between effects recorded under fasting and feeding conditions

Endpoint	Time	Average	SD	Min	Max
Mortality	24	NC	NC	NC	NC
	48	0.8	0.5	0.0 (PP)	1.0 (S; PE + S; PVC + S)
	72	1.8	1.0	0.0 (PVC)	3.3 (PVC + S)
	96	3.0	1.8	1.4 (PE + S)	6.0 (PVC/PE + S)
Immobilization	24	0.9	0.5	0.0 (PP)	1.3 (S)
	48	1.7	1.0	1.0 (PP; PVC; PVC + S)	4.0 (PE)
	72	1.7	0.5	1.0 (PVC)	2.3 (PVC/PE + S)
	96	2.0	0.7	1.2 (PVC + S)	3.0 (PE)

Average ratios (pure numbers) of effects recorded under fasting and feeding conditions are reported for each exposure time (including standard deviations, minimum, maximum values, and substance/s associated). As example, the ratio between observed average effects of 40% and 20% gives a value of 2; while the ratio between 0% and a number gives not calculable value (NC). As consequence, ratios = 1.0 mean that recorded effects under fasting and feeding conditions are equal. Ratios > 1.0 mean that effects are higher under fasting conditions. Ratios < 1.0 mean that effects are higher under feeding conditions. Animals exposed under feeding conditions show the higher mortality values compared to fasting conditions till 48 h. Starting from 72 h under fasting conditions effects are 1.8–3.0 folds higher than under feeding conditions. Concerning immobilization, starting from 48 h effects recorded under fasting conditions are 1.7–2.0 folds higher than under feeding conditions

(PE + S = 26.7% vs. S = 22.2% and PE = 6.7%). PVC results are similar to PE. On the contrary, PP + S and PVC/PE + S, provide evidence of a reduction of toxicity compared to PP and PVC/PE dispersions without the addition of surfactant. After 96 h, the toxicity of PVC + S is the highest. Table 2 reports the effects of animal aging on toxicity. Mortality shows higher values (ranging within 1.7%–31.7%) exposing aged animals than early stages. On the contrary, concerning immobilization, fasting conditions produce higher effects on aged animals while, under feeding conditions, the differences recorded between effects according to the age of the animals are, on average, lower.

Table 2 Effects of aging on recorded toxicity

(a) Mortality (%)		Neonates				Aged animals			
		PE	DS	PVC	DS	PE	DS	PVC	DS
Feeding condition	24 h	0.0	0.0	0.0	0.0	0.00	0.00	6.67	11.55
	48 h	6.7	11.5	6.7	11.5	0.00	0.00	13.33	23.09
	72 h	20.0	20.0	20.0	20.0	20.00	0.00	40.00	20.00
	96 h	33.3	30.6	53.3	30.6	46.67	11.55	93.33	11.55
Fasting condition	24 h	0.0	0.0	6.7	11.6	6.7	11.6	0.0	0.0
	48 h	6.7	11.6	6.7	11.6	26.7	23.1	20.0	0.0
	72 h	33.3	23.1	66.7	30.6	80.0	34.6	66.7	30.6
	96 h	46.7	11.6	80.0	20.0	100.0	0.0	80.0	20.0
(b) Immobilization (%)		Neonates				Aged animals			
		PE	DS	PVC	DS	PE	DS	PVC	DS
Feeding condition	24 h	6.7	11.5	0.0	0.0	6.7	11.6	20.0	11.6
	48 h	26.7	20.0	33.3	30.6	13.3	11.6	46.7	23.1
	72 h	40.0	20.0	66.7	11.5	40.0	0.0	73.3	23.1
	96 h	46.7	11.5	66.7	11.5	53.3	11.6	93.3	0.0
Fasting condition	24 h	6.7	11.6	6.7	11.6	20.0	23.1	40.0	20.0
	48 h	33.3	11.6	33.3	57.7	80.0	11.6	73.3	23.1
	72 h	46.7	11.6	80.0	23.1	93.3	23.1	86.7	20.0
	96 h	73.3	11.6	80.0	20.0	100.0	0.0	93.3	11.6

Effects recorded are referred to the exposure to water dispersions of microplastics + surfactant (S; Triton X-100). Two microplastic types tested are PVC and PE. Neonates are early stages according to OECD (2004) guideline while aged animals are 10 days old at the beginning of the exposure experiment. Negative controls are not reported but recorded trends were similar to values reported in Fig. 2 and Fig. 3. Two different endpoints are reported

DS standard deviation

Small-sized microplastics can produce mechanical damages as impair of filtering activities, affecting gut integrity, and translocating from gut into tissues (Cole et al. 2013; Rehse et al. 2016; Ma et al. 2016). In this study, we recorded the formation of homo-agglomerates of microplastics, which adhere on animals' body and strongly affect their fitness by trapping them, reducing their motility and increasing the energy consumption spent to break themselves free from homo-agglomerates of microplastics. *D. magna* is a filter feeder foraging non-selectively on particles within a size range of < 1–70 µm (Ebert 2005; Rosenkranz et al. 2009; Rehse et al. 2016; Nørgaard and Roslev 2016; Frydkjær et al. 2017). In this study, we recorded direct ingestion of all tested microplastics types (10–100 µm size) under both fasting and feeding conditions. Observed ingestion was also reported by the literature. According to Frydkjær et al. (2017), animals exposed to regular and irregular polyethylene (PE) microplastics at doses within 0.0001–10 g/L, ingested 0.7–50 particle/animal/day.

Our results on negative controls provide evidences as feeding conditions consent to record significant effects during longer exposure tests (96 h). Under fasting conditions acceptable results sensu OECD (2004) were recorded not later than 24 h. After 48 h, average mortality was near to 10% while immobilized animals exceeded this limit value. Also, standard deviations become larger affecting significance of observed results (OECD 2004). Feeding allows performing long-time exposures evidencing, till 96 h, more stable results with mortality and immobilization averages < 10% and narrow standard deviation ranges. Immobilization represents a more sensitive endpoint compared to mortality to evaluate early effects due to the exposure to microplastics. These results also support the results obtained by the recent literature (Imhof et al. 2017).

We tested higher concentrations of microplastics than levels reported for natural freshwater ecosystems (ranging within ng/L-µg/L; Lassen et al. 2015; Duis and Coors 2016; Fischer et al. 2016).

Nevertheless, our results provide evidences that microplastic affects mortality and immobilization on exposed animals under both fasting and feeding conditions. It could be useful to better understand and eventually to size further researches on sub-lethal. The ingestion of microplastic depends on several factors and could be affected by plastic type (Wright et al. 2013) producing the different effects recorded in this study.

In regards to ecotoxicity recorded in our research, some materials were never tested before such as PP, PVC, PVC/PE and their mixtures with surfactants. In Europe, results recorded after 24 h of exposure under fasting conditions (OECD 2004) are used to evaluate H14 risk (ecotoxicity for the aquatic environment). Concerning PE, exposure under fasting conditions, produced 30% of immobilized animals (at 48 h and 0.05 g/L). Our result on PE is comparable to recent literature reporting a higher toxicity for irregular shaped microplastics (EC50 = 0.065 g/L) compared to regular ones (EC50 = 5 g/L) (Frydkjær et al. 2017). The difference of about 20% is due to the 2 h feeding performed in our study before starting the long-term exposure experiments and to the 0.015 g/L lower dose of exposure compared to Frydkjær et al. (2017). After 96 h, the immobilization recorded in this study resulted to be on average 60% (PE). This value is 10% higher than the values reported by Rehse et al. (2016) for particles of approximately 1 µm of diameter under the same experimental conditions.

Differences recorded in this study among tested microplastic types could be due to their different densities. In fact, PE (915–930 kg/m^3; Pavan and Frassine 2005), and PP (900–1050 kg/m^3) densities allow them a major availability for *D. magna*; on the contrary, PVC density is higher (1350–1420 kg/m^3; Patrick 2006) favouring PVC sedimentation and reducing availability for *D. magna*.

Surfactants are widespread in aquatic environments and are widely recorded at high levels mainly in effluents from municipal wastewater treatment plants (Renzi et al. 2012) where surfactants could be present associated to microplastics (Murphy et al. 2016). Surfactant tested in this study shows an effect on *D. magna* by increasing its mortality and immobilization of the exposed individuals. The addition of the surfactant to microplastic particles and the dispersion of it produce new effects on recorded mortality and immobilization of tested microplastic types. Our results suggest an increased toxicity of microplastics when surfactants are present, nevertheless recorded exceptions evidenced that toxicity associated to microplastic/surfactant dispersions depends on the type of microplastics. Mechanisms of action could be different. For example, PVC increases toxicity when animals are exposed to PVC + S, while the toxicity of PP is not affected by the surfactant, probably due to the better chemical resistance of PVC (Wypych 2015). Furthermore, it is known from the literature that microplastics could represent a vector for chemicals exerting toxicological effects on zooplankton after ingestion (Ziccardi et al. 2016). A recent research provides evidence that the addition of phenanthrene to dispersions of irregular shaped microplastic reduces EC50 of about 3.4 folds on *D. magna* after the exposure under fasting conditions (Frydkjær et al. 2017). Sorption dynamics among surfactant and different microplastic types show effects in the modularization of ecotoxicity. As well as hydrocarbons, surfactants could be absorbed from the environment onto the microplastic surface and could be successively released inside the digestive system of the animals (Teuten et al. 2007).

Further studies are needed to better understand the mechanism of action and the factors that are able to modulate toxicity and toxicity magnitude of surfactants and microplastic dispersions (i.e. physical and chemical interactions, routes of assumption). The interactions among microplastic types and surfactants in natural environments could produce effects on aquatic species and fasting or feeding conditions could affect animals' responses (Jemec et al. 2016). In this study, trends under fasting and feeding conditions occur at different orders of magnitudes suggesting that some sorption process modulated by food stuffs are occurring under feeding conditions. This result agrees with the literature that reports the occurring of interferences among chemical sorption processes and algae concentrations in medium (Frydkjær et al. 2017). In rivers and in lakes both the algal concentration and algal/microplastic ratios could change significantly by a factor of 10^3 (Frydkjær et al. 2017). For all types of microplastic tested in our study, lower effects under feeding conditions are recorded. For example, the toxicity recorded for PE under fasting condition was 4 times higher than what recorded under feeding conditions.

Our study provides evidence that the level of toxicity of micro plastics depends on the age of the animals. The different surface tension of different microplastic types could affect early developmental stages in presence of surfactants. PE shows a higher surface energy than PVC and could not be faced by neonates probably because they have not enough energy, especially under fasting conditions, to get rid of them. PVC + S results the most toxic among tested dispersions for both neonates and aged animals especially under fasting conditions probably due to the non-selective feeding habits of *D. magna* (Rehse et al. 2016).

In conclusion, our results clarify some key aspects on the toxicity of tested microplastic types such as the effects on toxicity related to the presence of surfactants, the feeding condition, and the aging but further researches will be performed. Further studies are needed to better clarify some key aspects reported by our research. In particular, effects at microplastic doses similar to environmental ones will be further explored to better understand real risks for freshwater ecosystems. Relationships among microplastic types and surfactant sorption/desorption processes should be further clarified. Different species show different sensitivity to toxicants (Lechuga et al. 2016) and further data on a wider range of aquatic species should be collected in order to perform a risk evaluation on aquatic environments.

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