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Ecotoxicological effects of atmospheric particulate produced by braking systems on aquatic and edaphic organisms

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ABSTRACT

Vehicles generate particulate matter (PM) in significant amounts as their brake systems wear. These particles can influence air quality and their transport/deposition may affect the edaphic and aquatic ecosystems. As part of the LOWBRASYS H2020 project, new more eco-friendly brake disc and pad formulations were developed. PMs generated from traditional (FM1-BD1) and innovative (FM4-BD2, FMB-BD7) brake systems in bench tests were studied. The PMs' physical/chemical characteristics were preliminarily investigated. To study the possible environmental impact of the nano-micro particulate, we used a battery of ecotoxicological tests. We employed the microalga *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the bacteria *Vibrio fischeri* as aquatic bioindicators, while for the edaphic ecosystem we used the seeds of *Lepidium sativum* and *Sorghum saccharatum*, the nematode *Caenorhabditis elegans*, the earthworm *Eisenia andrei* and the ameba *Dictyostelium discoideum*. The results showed a higher sensitivity of the freshwater organisms exposed to the soluble PM fraction, with respect to the edaphic ones. FM4-BD2 brake formulation was slightly more toxic for algae (200 mg/L) than FM1-BD1 (500 mg/L). The new system FMB-BD7 particulate was not harmful for crustacean survival, and resulted weakly toxic for algal reproduction only at 500 mg/L. The particulate material per se was found to affect the algal reproduction. No toxic effects were found on nematodes, earthworms and seeds up to 1000 mg/L. However, in *D. discoideum* the reproduction rate was significantly reduced starting from 100 mg/L; and the lysosomal membrane stability showed a relevant alteration also at minimal concentration (0.1 mg/L). The results demonstrated a minimal risk for biodiversity of the particulates from the different brake systems and highlighted a more eco-friendly performance the new brake-pad FMB-BD7. However, the occurrence of sublethal effects should be considered as a possible contribution of the particle toxicity to the biological effects of the environmental pollution.

1. Introduction

Emissions from vehicles are an important source of environmental contamination. The particulate matter (PM) produced by road transport systems is a consequence of exhaust emissions (contributing to the fine fraction of the atmospheric particulate) and also the result of non-exhaust emissions originating from road surface abrasion and mechanical wear of clutches, tires and brakes (Kam et al., 2012; Wahid 2018; Perricone et al., 2019). These latter have attracted the attention of the scientific community (Ketzel et al., 2007; Harrison et al., 2011, 2012; Denier van der Gon et al., 2013) which has remarked their impact on air quality, human and environmental health. Brake wear can contribute up to 50% by mass to total PM_{10} emissions in urban settings (Harrison et al., 2012; Lawrence et al., 2013), decreasing in highway driving due to lower braking frequency. The PM generated is generally composed of nanoparticle aggregates (Grigoratos and Martini, 2014) that facilitate the concentration of contaminants, heavy metals and carbon nanostructures (Wåhlin et al., 2006; Amato et al., 2011; Varrica et al., 2013). There is very little information on their related biogeochemical behaviour and these gaps derive partly because of the extreme heterogeneity of this kind of particulate and the processes that lead to its formation (Kukutschová et al., 2011). Therefore, the industrial development of new solutions to ensure more eco-friendly braking systems with better emission performance in terms of mass, composition and particle

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number and size, could make a significant contribution to improving air quality.

Within the LOWBRASYS project, funded by the EU Horizon-2020 program, the partners involved have tested tools, materials and driving solutions to achieve a considerable reduction (at least 50%) of brake PM emissions. The main technologies focused on testing novel solutions for brake pads and discs in specialized bench tests. Among the many formulations tested, the two best in terms of performance, composition and improvement in PM emissions (which will be referred to as FM4-BD2 and FMB-BD7) were selected and compared to a standard (FM1-BD1), already on the market, to establish the differences in the resulting PM and assess their potential toxicity. The brakes' particulate daily deposits and accumulates in urban dust and its transport and subsequent redeposition (Glišović et al., 2018) can convey it at considerable distances, increasing the pollution load in water and soil.

The aim of this study, as part of the broader LOWBRASYS program, was to investigate the effects of brake wear particles in aquatic and edaphic environments, using several ecotoxicological assays. The battery of acute and chronic tests was selected on the basis of the ecological representativeness of models belonging to the primary production and the grazing and detritus trophic chains. The choice of the most ecologically relevant model organisms is critical to obtain realistic results. For this study, we selected the unicellular green alga *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the aquatic bacteria *Vibrio fischeri* as bioindicators of the aquatic environment; the seeds of mono- and di-cotyledons (*Sorghum saccharatum* and *Lepidium sativum*), the pore-water living amoeba *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans* and the Oligochaete earthworm *Eisenia andrei* were used for the edaphic compartment. The effects at organism/population level were investigated through high-level endpoints including bioluminescence (*V. fischeri*) and growth inhibition (*P. subcapitata*, *D. discoideum*), mortality (*D. magna*, *C. elegans*, *E. andrei* and *D. discoideum*) and seed germination (*L. sativum*, *S. saccharatum*). As a link between the early cellular effects at the sublethal level and the high-level endpoints, we tested the physiological response to stress using as biomarker the lysosomal membrane stability (LMS) on *D. discoideum*, sensitive to toxicant-induced effects even at sublethal level (Sforzini et al., 2008).

In accordance with the toxicity parameters expected for chemicals that are hard to test in the aqueous phase, the ecotoxicological assays on aquatic organisms were done using the bioavailable soluble PM fraction or water accommodated fraction (WAF). Any effect of the PM in water is manifested as toxicity transient during the deposition, so further analyses were done by exposing the organisms directly to brake-wear particulate (BW-PM) in aqueous suspension, using two types of quartz sand in a comparable size range as laboratory controls.

To assess the actual impact and the potential risk of BW-PM dispersion in the environment, the concentrations tested were compared to the BW-PM fallout estimated using an up-to-date 3D Lagrangian modeling system in a real extra-urban environment including edaphic and aquatic compartments and crossed by a highway.

2. Materials and methods

2.1. Bw-pm collection and storage

Brake-wear particulate matter (BW-PM) for commercial and innovative brake disc and pads samples, were collected during specific inertia brake dynamometric tests. A full-scale dynamometer adapted to measure the particle emission was used to generate and collect wear dust (Perricone et al., 2016). The test procedure is a sub-section of the industry-used Los Angeles City Traffic (LACT) test cycle, called 3 h-LACT, comprising 217 stops, simulating actual driving conditions (Mathissen and Evans., 2019). Three different pairs of brake pads were used during the experiments with a cast iron brake disc: one reference disc and pads already on the market (FM1-BD1) and two innovative ones (FM4-BD2 and FMB-BD7). A pooled sample was generated with all three mixing PM_{10} particles and coarser fractions collected during the bench tests. X-ray fluorescence spectroscopy (XRFS) was used to analyse the chemical composition of the brake linings of the pads.

2.2. Water accommodated fraction (Waf)

The water accommodated fraction (WAF) is an aqueous medium containing only the fraction of the substance that is dissolved or present as a stable dispersion or emulsion (Organisation for Economic Co-operation and Development, OECD., 2000). The powder suspensions were sonicated for 1 h then left under shaking for 24 h at room temperature (about 20 °C). The solutions were centrifuged at 500 rpm for 5 min then filtered on a 0.2 µm filter (Organisation for Economic Co-operation and Development, OECD., 2002).

2.3. Bw-Pm Physical/Chemical characteristics and size distribution

A scanning electron microscopy-(SEM) (FEI ESEM Quanta 200, with attached EDAX electronic microwave) was used to analyse the morphological characteristics of wear particulate with Genesis interface software. The observation was done in low vacuum mode, with a variable pressure in a chamber equal to 90 Pa, without any further manipulation. The analyses were carried out at 20 kV acceleration voltage, 15nA beam current and a working distance of about 10 mm. The energy dispersive X-ray spectrometry (EDS) to determine the chemical composition were carried out considering as fixed parameters 80 L sec and a Death Time between 15 and 35%.

The particle size and the presence of agglomerates and aggregates were analyzed by Dynamic Light Scattering (DLS). Measurements were done with a ZetasizerNanoZS (Malvern Instruments) operating in a particle size range from 0.6 nm to 6 µm, equipped with a He-Ne laser with λ633 nm. Before analysis, samples were dispersed in ultrapure water and the dispersions were stirred at room temperature for 10 min, then sonicated for another 10 min.

2.4. Ecotoxicological analyses

2.4.1. Growth inhibition of P. subcapitata

The bioassay followed the standard ISO, 8692:2012 (Water quality-freshwater algal growth inhibition test with unicellular green algae) using the unicellular Chlorophycea *P. subcapitata*, ubiquitous in fresh water and easily grown in the laboratory. Algal cultures in exponential growth phase (1×10^6 cells/mL) were used. Growth inhibition was measured after 72 h exposure to samples.

In a parallel set of experiments, a test was done to verify the algal growth inhibition by exposing the organisms directly to the brake particles, using as control two types of quartz sand of different granulometry in a size range comparable to that of the PM resulting from the wear of braking systems (BCR-066: quartz 0.35–5 µm; silica glass spheres 9–13 µm). WAF, BW-PM and quartz sand suspensions were prepared, using the specific nutrient stock solution (in MilliQ water) as laboratory control. Algal solution (20 µL) was inoculated in sterile 24-well plates with 2 mL of each treatment (from 0.1 to 1 mg/L) for 3 days at 25 ± 1 °C in a thermostatic chamber. Algal density was measured at the beginning and end of treatment with a hemocytometer chamber (Bürker).

2.4.2. Acute test of immobilization on D. magna

The immobilization assay on *D. magna* was used to evaluate the acute aquatic toxicity of the BW-PM. The crustacean bioassay was done following the standard ISO, 6341:2012 (Water quality – determination of the inhibition of the mobility of *D. magna* Straus (Cladocera, Crustacea)–acute toxicity test). For each replicate, ten daphnids were exposed to 50 mL of treated solutions (WAF, BW-PM and Quartz Sand) at the concentrations previously described for 24 and 48 h at 20 ± 2 °C, with a 16/8h light–dark cycle. Their immobilisation/death was recorded and compared with control values.

2.4.3. *V. fischeri* bioluminescence inhibition (Microtox®) test

The bioassay with the luminescent bacterium *V. fischeri* followed the standard ISO 11348-3:1998 (Water quality – determination of the inhibitory effect of water samples on the light emission of *V. fischeri* (luminescent bacteria test) - part 3: method using freeze-dried bacteria). The reduction of light emitted after 15 min exposure to samples was recorded with a Microtox® luminometer.

2.4.4. Germination rate and root elongation of *L. sativum* and *S. saccharatum*

Acute toxicity to terrestrial plants was investigated with the phytotoxicity test following the method UNICHIM No. 1651 (2003), which provides for the use of *L. sativum* (a dicotyledonous species) and *S. saccharatum* (a monocotyledonous species). Their seeds were grown in artificial soils (quartz sand and standard OECD soil −5% peat, 10% kaolin and 85% sand), used as lab controls, to which increasing concentrations of BW-PM obtained from FM1-BD1, FM4-BD2 and FMB-BD7 formulations were added.

For each replicate, an amount of 10 g (dry weight) of soil per plate was placed in Petri dishes and moistened with 5 mL of sample dilutions in MilliQ water; Whatman #1 filter paper was then put on the wet soil and ten seeds were randomly placed on the filter. The plates were incubated for 72 h in the dark at 25 ± 2 °C. After incubation, the germinated seeds with root length ≥ 1 mm were measured, and data from each plate were used to derive the germination index (GI). The test is considered valid if the seed germination in the lab control is > 90%. For the WAF samples, a Whatman #1 filter paper was placed in a Petri dish and 5 mL of the sample was added. Then, ten seeds for each species were put on the filter.

2.4.5. Toxicity test on the nematode *C. elegans*

The acute toxicity test (Ura et al., 2002) involves exposing the synchronous organisms (to prevent age from affecting their response to exposure) to the WAF samples for 24 h at 20 °C. Ten one-day-old worms were dispensed into 24 well culture plates containing 0.5 mL of K-medium (32 mM KCl, 51 mM NaCl) and exposed to different BW-PM concentrations, without feeding the organisms. At the end of the incubation period, live nematodes were counted.

2.4.6. Toxicity test on the soil earthworm *E. andrei*

Earthworms were cultured as described in the OECD guidelines (OECD, 1984; OECD, 2004)in a breeding medium (at 20 ± 1 °C) made up of a mixture of horse manure and peat. Organisms were selected from a synchronized culture with a homogeneous age structure. Adult worms with clitellum of similar size and weight (400–500 mg) were used. The Filter Paper test was done as described in the OECD guideline for testing chemicals (OECD, 1984). Worms were kept on clean moist filter paper for 3 h before being placed in test dishes to allow them to void their gut contents. They were then washed with deionised water and dried before use (Sforzini et al., 2017). One mL of the BW-PM suspension was spread on a filter paper (Whatman #1) which was placed on the bottom of a Petri dish. Control filter papers were treated with 1 mL of deionized water. The dishes were put in a climatic chamber at 20 ± 1 °C. The test was done in the dark and for 48 h and 72 h. At least ten replicates per treatment, consisting of one worm per dish, were used. At the end of the incubation period, live earthworms were counted.

2.4.7. Acute and chronic tests on *D. discoideum*

The amoebic cells were cultured as described by Sforzini et al. (2016) until their concentration reached 2–4 × 10^6 cells/mL. After centrifugation, the cells (0.75 × 10^6 cells/mL) were incubated for 3 h and 24 h in the samples (25% AX-2 medium with 50 μM $CaCl_2$ and 10 μg/mL tetracycline) to assess their vitality and reproduction rate, respectively. Cell viability was estimated incubating the cells with propidium iodide (Invitrogen Molecular Probes, Eugene, OR, USA); cells were observed at 200 × magnification (Zeiss Axio Observer) using a rhodamine emission filter (setting and transmission light simultaneously) to distinguish dead cells (with a fluorescent nucleus) from living ones. Reproduction rate was assessed using a hemocytometer chamber (Bürker).

Lysosomal membrane stability (LMS) was evaluated after 3 h of exposure as described by Sforzini et al. (2016). Amoebae were put in adhesions on a glass coverslip for 10 min inside a humidity chamber at 21 ± 1 °C. The cells were then added in a working solution of Neutral Red (NR), obtained by diluting a stock solution of NR (20 mg of NR in 1 mL of dimethyl sulfoxide) 1/800 in Page's Amoeba Saline (PAS) solution. After 3 min incubation, the dye in excess was removed, and the cells were washed and kept moist with PAS. The retention time of NR dye in the lysosomes was checked after 1 h. Slides were observed under 400× magnification with an inverted photomicroscope (Zeiss Axio Observer) equipped with a rhodamine emission filter for fluorescence investigations. Images were analysed with the Scion Image analysis system, for the quantification of lysosomal NR leakage, expressed as a percentage change in fluorescence intensity with respect to the controls.

2.5. Statistical analysis

For all the different tests used in this research, we analysed at least four replicates from controls and BW-PM exposed organisms. Due to the number of data available for the statistical significance evaluation of the biological effects produced by the different BW-PM concentrations, we employed the non-parametric Mann-Whitney *U* test to compare the data from treated organisms with those of the controls ones ($p < 0.05$).

3. Results

The biological effects of the WAF and BW-PM from FM1-BD1, FM4-BD2 and FMB-BD7 brake disc and pad formulations were studied using bioassays with model organisms of aquatic and terrestrial ecosystems. The possible effects at organism/population level were investigated using high-level endpoints such as survival and/or reproduction rate.

For the physical characterization of the particulate debris, the DSL technique was employed to identify the dimensional range of the three BW-PM samples from the changes in the intensity of the diffused light. The results indicated a tendency to aggregation of the PM as a function of time (the three successive continuous scans were taken every two minutes), as easily seen from Fig. 1.

There were several sub-micrometric particles with a tendency to aggregation as evidenced by the arrows in Fig. 2, acquired in the FM1-BD1 sample. A morphology of fibrous nature was evident in Fig. 2a, indicated by the green arrow. The tendency to particle aggregation was even more evident at higher magnifications; in Fig. 2b the particles clearly aggregate in micrometric clusters.

The percentages obtained by chemical analysis (Table 1) showed iron in the majority, together with oxygen, suggesting a significant amount of iron oxides in the wear particles. There were also found substantial amounts of Cu, Zn, Sn, Cr, Al, Si, and S.

As concerns the ecotoxicological effects on the freshwater compartment, the microalga *P. subcapitata* was sensitive to the WAF from the particulate at concentrations higher than 100 mg/L, for two of the three formulations tested. The WAF solutions of one of the innovative pads (FM4-BD2) was weakly toxic at 200 mg/L (−40%) while the new formulation FMB-BD7 had the least effect on algae reproduction, since even at 500 mg/L the replication rate was reduced only by about 41% (Fig. 3a).

These results change significantly after exposure of the algal

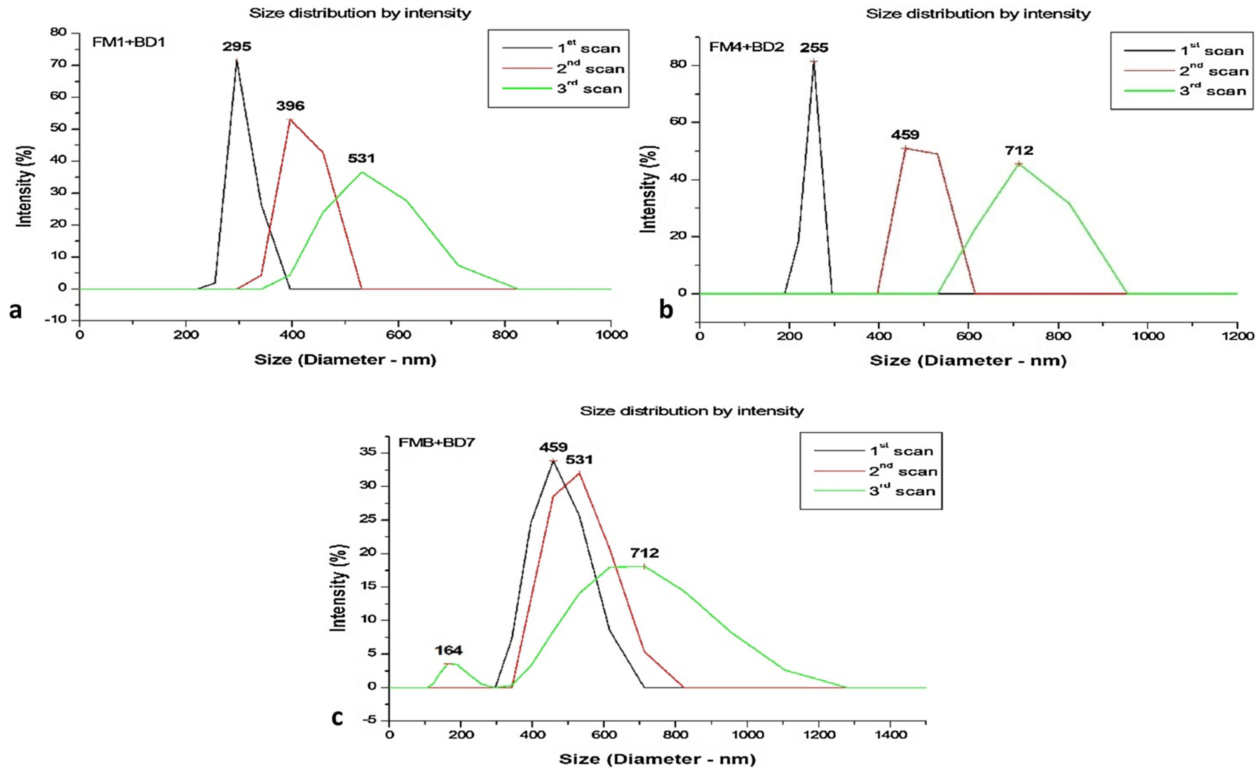

Fig. 1. Particles size distribution by intensity obtained from the three brake-system particulate, FM1-BD1 (a), FM4-BD2 (b) and FMB-BD7 (c) as measured by DLS technique.

inoculum to the BW- PM, to simulate the potential interaction with the organisms in the water column during the transient of the particles deposition towards the bottom. Fig. 3b shows that the particulate debris of the FM1-BD1 and FM4-BD2 formulations had a clear toxic effect already at the concentration of 1 mg/L while FMB-BD7 in the same conditions was only weakly toxic with evident effects at 100 mg/L.

Quartz sand used as laboratory control at size of 0.3–5 µm (BCR-066) started to show a mild toxic effect at 200 mg/L. Glass spheres (9–13 µm) showed a tendency more similar to that of the FMB-BD7 suspension up to 10 mg/L, above which (100 mg/L) algal vitality was almost completely zeroed (Fig. 3c).

The same experimental set-up was employed for the acute toxicity tests on the water flea *D. magna*. The WAFs obtained from FM1-BD1 and FM4-BD2, had clear toxicity only at concentrations higher than 100 mg/L, while the exposure of the crustacean to FMB-BD7 did not inhibit its motility (Fig. 4a). FM1-BD1 and FM4-BD2′ BW-PM proved more toxic (as low as 10 mg/L) but even in this case the PM of the FMB-BD7 formulation had no effects on the crustacean's survival (Fig. 4b), neither did the solutions from the two different types of quartz sand (data not shown).

In addition, the WAF solutions from all the brake pad formulations were not toxic for *V. fischeri* up to 1000 mg/L and, again, FMB-BD7 gave the most eco-friendly values (Fig. 5).

The results about the BW-PM' effects on the edaphic organisms clearly demonstrate that the WAF solutions have not affected the germination of mono-dicotyledonous seeds (*S. saccharatum* and *L. sativum*)

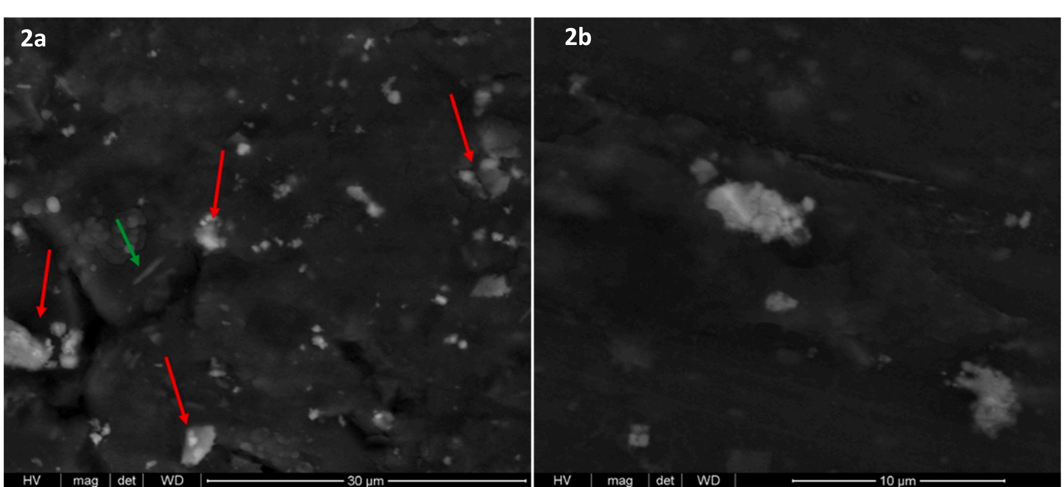

Fig. 2. SEM images in secondary electrons acquired on an area of the sample FM1-BD1 (a), and at higher magnification (b). The sub-micrometric particles' tendency to aggregate was reported (red arrows). A fibrous morphology was also found (green arrow).

Table 1
Elemental composition obtained from the three brake-system particulate. Data are expressed as percentage of weight. "–" indicates that the element is below the limits of detectability.

Chemical species	FM1-BD1 [w/t%]	FM4-BD2 [w/t%]	FMB-BD7 [w/t%]
O	26,13	23,36	27.67
C	11,82	9,04	11.95
Fe	42,96	31,7	39.05
Mg	1	1,56	2.67
Al	1,72	2,99	1.82
S	0,77	1,22	2.22
Ba	–	–	5.33
Ca	1,87	0,5	0.12
Cr	0,99	1,44	1.34
Sn	–	2,72	4.24
W	–	1,6	2.28
Si	1,23	1,68	0.33
Mn	0,32	0,26	0.21
Zn	3,1	5,54	0.74
Cu	7,99	14,98	0.06
Ti	0,1	1,41	–

at the highest concentration tested of 1 g/L. Instead, the effects of these particulates in only quartz sand were evident from 500 to 1000 mg/kg for FM1-BD1 and FM4-BD2. Only FMB-BD7 did not affect the germination of the seeds; however, the BW-PM had no negative effects when the two seeds were exposed in more natural conditions, i.e. in standard OECD soil (Fig. 6).

The same concentrations had no toxic effects on the nematode *C. elegans* or on earthworm *E. andrei* during a standard OECD three-day filter paper test (data not shown).

For the high-level endpoints, the results with the BW-PM formulations on the pore-water amoeba *D. discoideum* showed that the particulate did not affect cell viability (Fig. 7a) even at the highest concentration used (1000 mg/L). However, the chronic toxicity test data indicated a significant decrease in the reproduction rate of the protozoan (Fig. 7b), caused by FM1-BD1 and FM4-BD2 BW-PM at 100 mg/L; in the same conditions, FMB-BD7 had slightly less inhibitory effect on cell division.

The evaluation in the amoebae of LMS, a very sensitive stress biomarker, showed that all BW-PM formulations caused a significant decrease in fluorescence intensity, derived from the NR staining of the lysosomes (respectively equal to 35% for FM1-BD1, 47% for FM4-BD2 and 31% for FMB-BD7), starting from the least concentration i.e. 0.1 mg/L (Fig. 8).

The results of the dispersion model were clarified in a parallel study (manuscript in preparation). This shows that in the extra-urban environment dry deposition related to the FMB-BD7 BW-PM_{10} fraction was 27.95 mg m-2 year-1 at the roadside (i.e. approximately 20 m from the side of motorway) and decreased 7 and 30 times at respectively 200 m and 500 m from the side. These values are (on average) 56% and 43% higher than the depositions produced by FM4-BD2 and FM1-BD1 respectively (Table 2).

4. Discussion

Particulate from braking (i.e. friction between the brake disc and pads), can interact, due to atmospheric dispersion and deposition, with aquatic and edaphic ecosystems. It is therefore impossible to neglect the potential eco-toxicological impact, which is substantial in terms of the quantitative representativeness of the PM contamination and the presence of heavy metals and organic contaminants in each different formulation of braking devices (Thorpe and Harrison, 2008; Plachá et al., 2017). This explains the current focus on sustainable brake formulation in terms of the composition of materials and abatement of emissions. We investigated the ecotoxicity of wear particles from commercial

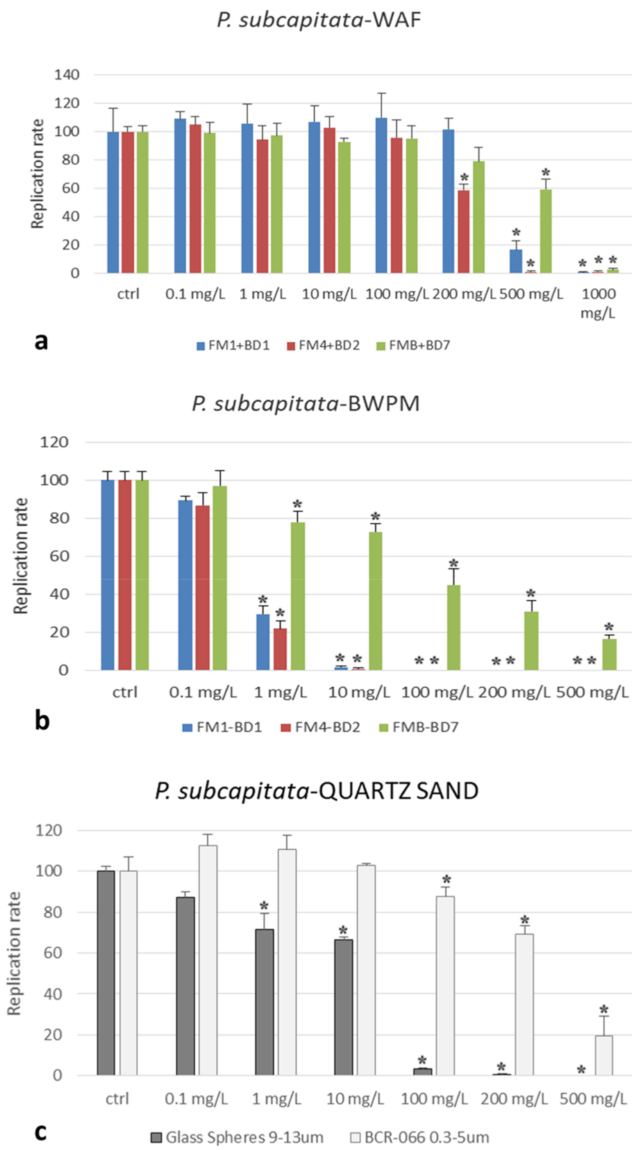

Fig. 3. (a-c). Growth of the alga *P. subcapitata* after exposure respectively to the WAF (a) and BW-PM (b) and from the three brake-system particulate, and tothe different size quartz particles (9–13 µm and 0.3–5 µm)solutions (c). Data represent the mean ± SD of at least four replicates: *statistically significant differences with respect to control values (p < 0.05 Mann-Whitney *U* test).

brake and new, supposedly more eco-friendly formulations proposed within the LOWBRASYS project. We used organisms typical of aquatic and soil ecosystems. Since the potential risks of nano-micro particulate can be related to the shape and size of the particles and to their chemical composition (Nel et al., 2006), the first step was the physico-chemical characterization of the particulate. DLS analysis, in combination with the SEM images, enable us to determine the particle size distributions in aqueous solutions. There was a tendency to aggregate over time with increasing size, indicated by the succession of DLS scans. The SEM images, confirmed by optical microscopy, indicate that the PM is made of hundreds of nanometer particles, which agglomerate as a result of the interactive forces generated (Kukutschova et al., 2009). This can be an obstacle to interpretation of the results because some important physical parameters are influenced by the aggregation state (Van Hoecke et al., 2008). By crossing the distribution data with the SEM images, however, we obtained more information on both the particles' real dimensions and morphologies as well as the dynamics of their variations.

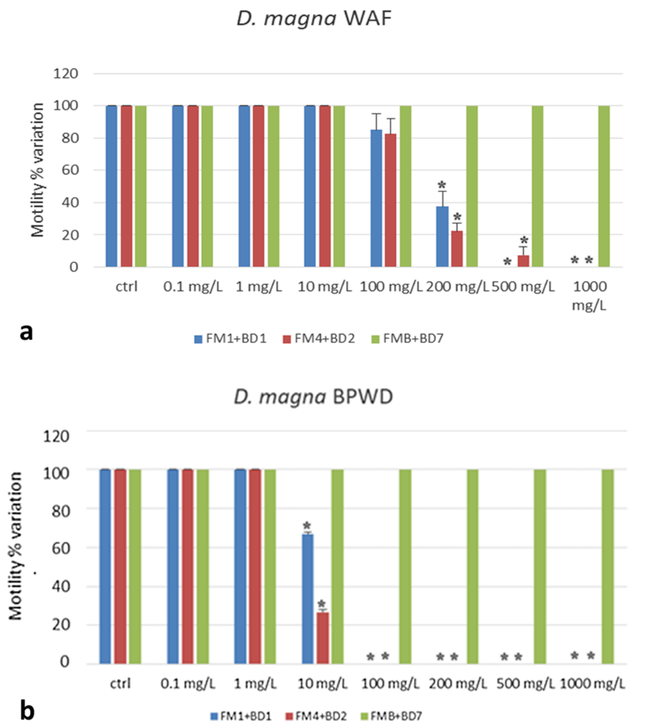

Fig. 4. (a-b). Motility of the crustacean *D. magna* after exposure to the WAF (a) and BW-PM (b) from the three brake-system particulate. Data represent the mean ± SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney *U* test).

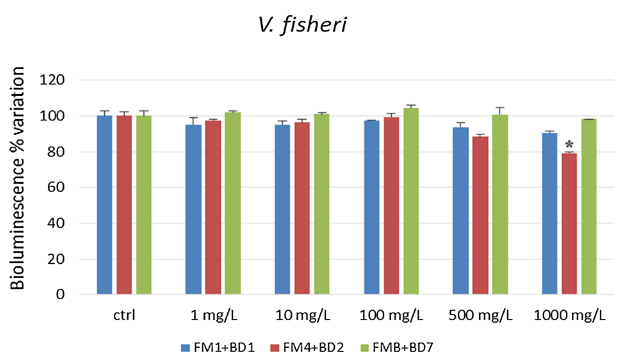

Fig. 5. Bioluminescence variation of the bacterium *V. fischeri* after exposure to the WAF from the three brake-system particulate. Data represent the mean ± SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney *U* test).

The chemical composition of the BW-PM showed the prevalence of metal oxides and elemental carbon forms, as reported in previous studies (Kukutschová et al., 2010; Kukutschová et al., 2011; Ciudin et al., 2014). For all the formulations Fe, in slightly different percentages, was the most representative element given that the discs are generally made of cast iron (Malachova et al., 2016). It is rare to find iron concentrations that induce environmental toxic effects; it is a limiting factor for the development of plants and animals and is toxic only under certain conditions (i.e. excess of Fe^{2+} and Fe^{3+} free ions, bioavailability within the matrix) and at very high concentrations. Even the iron oxides, present in large quantities in brake wear residues, are generally considered non-toxic. It is more important, however, that brake pads are the main source of environmental pollution due to copper, present on average in 1–14% of the total composition (Haselden et al 2006; Straffelini et al., 2015). Many studies have focused on this, because of the potential toxic effects of this essential metal (Viarengo et al., 1988; Leili et al., 2008; Kothai et al., 2008; Lee and Hieu, 2011) on human and environmental health, especially with regard to the quality of water (Armstrong 1994; Topping and Kuwabara, 2003).

Like Fe, Cu is an important component of the enzymatic systems of respiratory metabolism and photosynthesis, but is also a priority pollutant according to the European criteria (Italian Legislative Decree No. 172/2015). Vehicle emissions and brake pad dust (Drapper et al., 2000), together with widespread use in pesticides (USEPA 2005) and industrial processes (Good 1993; Thomas and Greene 1993), have mobilized significant amounts of copper metal, Cu-oxides and Cu-sulphides into the environment over time. The copper concentration in living organisms is partly controlled by homeostatic mechanisms but it becomes toxic if in overload and following long exposure (De Boeck et al., 1995; Lundebye et al., 1999). However, the toxicity, rather than the total copper content, is due to the readily bioavailable Cu^{2+} fraction, which in turn depends on the physical, chemical and biological characteristics of the matrix from which the release takes place (Viarengo and Nott, 1993; Burlando et al., 2004; Negri et al., 2013). The high content of copper particulate reaching the aquatic compartment through washout is noxious in the soluble Cu^{2+} form, as it may exert considerable toxic effects on algae and invertebrates (Kiaune and Singhasemanon, 2011), with possible repercussion on the aquatic food chain.

We found the chronic aquatic toxicity assay detected potential effects on *P. subcapitata* starting from WAF concentrations of 200 mg/L (mainly for the FM4-BD2 prototype), and therefore the ecological risk can be considered limited. In fact, it is extremely hard to reach these values in a lake or in the running water of a river. However, direct exposure of the algal inocula to the BW-PM, in order to simulate the potential interactions during the transient of the particles deposition towards the bottom increased the toxicity rate exercised by the three formulations, but in very different percentages for FM1-BD1 and FM4-BD2 compared to the most eco-friendly FMB-BD7.

The choice of quartz sand of various sizes as a laboratory control was aimed at ascertaining whether the particle sizes were a limiting factor for algal growth. Glass spheres with an average diameter of 9–13 µm do indeed drastically affect algal growth already starting from the concentration of 1 mg/L, while BCR-066 (0.3–5 µm) was perceptibly toxic only above 200 mg/L threshold, with results more closer to those for the BW-PM. According to Van Hoecke et al. (2008), although the quartz sand particles are not toxic by themselves, the SiO_2 solid colloid formation in a saturated solution may exert toxicity by clustering around the algal cells. Particles and aggregates with a diameter bigger than the microalgae cell-wall pores (from 5 to 20 nm, as reported by Navarro et al., 2008) cannot enter cells but may cause toxicity even through surface interactions, with direct effects on nutrient depletion, and/or shading, with consequent inhibition of photosynthesis (Van Hoecke et al., 2009; Ma et al., 2010).

However, the BW-PM of FM1-BD1 and FM4-BD2 were much more toxic than quartz sand, so the answer must be sought more in-depth.

Since the effects of the dissolved fraction of the BW-PM were less than those of the particulate itself, the toxicity might be due to the physical interactions of the particles themselves more than the release of free ions in solution. This, with the more pronounced aggregation due to the lack of sonication and filtering (unlike the WAFs) and the simultaneous combination of the above factors, may have contributed to affect algal growth. Even the classical acute toxicity test on *D. magna*, a model species particularly sensitive to heavy metals (Khangarot and Ray, 1989), detected much more evident immobilization/death effects for the FM1-BD1 and FM4-BD2′ BW-PM (10 mg/L), than the WAFs (200 mg/L).

Experimental evidence on the environmental interactions of vehicle wear particulates (Wik and Dave, 2009) suggests that the most likely route of particle uptake for filter-feeding organisms is through ingestion. *D. magna* has a range of active particle filtration approximately between 200 nm and 70 µm (Burns, 1968; United States Environmental Protection Agency., 2002), depending on the animal's size.

The toxicity mechanism, which for *P. subcapitata* was hypothesized to be mostly related to superficial physical–mechanical interference,

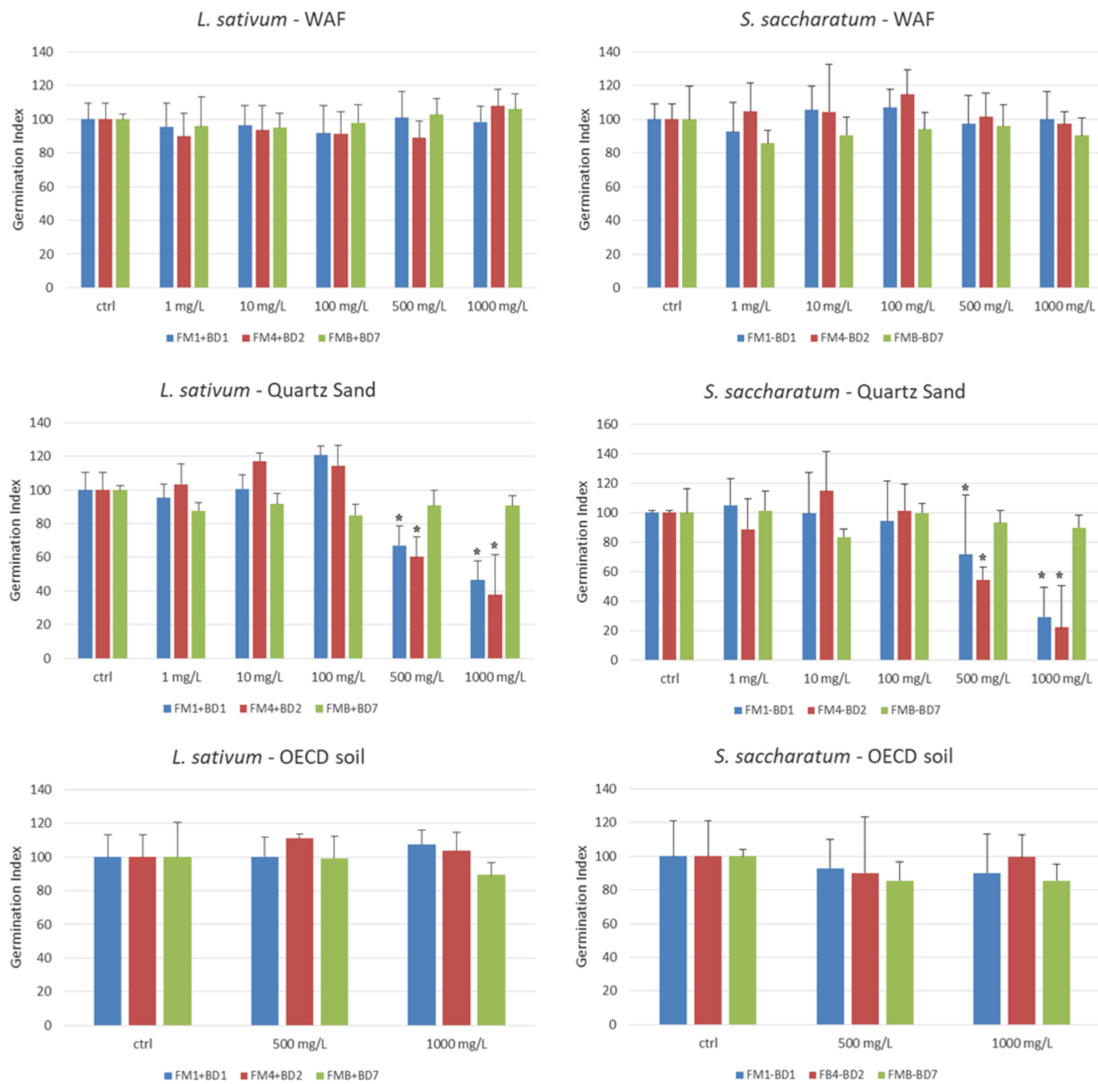

Fig. 6. Germination index of *L. sativum* and *S. saccharatum* seeds at different concentrations of the three brake-system particulate, respectively in WAF, quartz sand and OECD soil. Data represent the mean ± SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney *U* test).

has been linked in the case of *D. magna* to the processing of the actively ingested material. First, the size and the degree of aggregation of the particles may influence or interfere with their capture by the crustaceans' filtering apparatus. This may partly explain why the direct use of BW-PM increased the toxic effect: on the one hand, bulky aggregates may have compromised respiratory activity, and it has been shown (Peikertova and Filip, 2016) that nanoparticles produced by braking easily stick to the wear debris and after ingestion might be released inside the cellular compartments.

There are ample published reports on the severe toxic effects on freshwater cladocerans exerted by heavy metals and particularly by copper (Lewis, 1983; Vardia et al., 1988; Khangarot et al., 1987; Khangarot and Ray, 1987; Arambašić et al., 1995). In neutral aqueous solutions the dissolution of copper compounds is not facilitated (Wang et al., 2013) but during the passage in the gastrointestinal tract the enzymatic conditions would favour desorption even if the pH in the water flea's gut is almost neutral (Pennak, 1978). Then too, when internalised in the cells, these compounds could be picked up inside the lysosomal vacuolar system; there the acid pH (4–5) can break them down into free ions, enhancing their absorption, bioavailability and the consequent potential toxicity (Studer et al., 2010). If the lysosomal membranes become destabilized to the point of breaking, the intracellular release of free metal ions and their accumulation in the cytoplasm or the nucleus can be harmful (Viarengo et al., 1981; Brunner et al., 2006; Midander et al., 2009; Semisch et al., 2014).

Besides direct toxicity, another plausible hypothesis is that the particles seized within the digestive tract could react or interact with food compounds (microalgae and yeast), rendering them potentially useless for the organism (Rosenkranz et al., 2009). The acute test with *V. fischeri* did not allow the presence of particles in solution, which would have clearly interfered with the Microtox® detection system, but the results of the WAF did not show any particular toxic effect. This is particularly important in view of the role of the bacterial communities in the recirculation of organic matter in the sediments, which are the ultimate deposit of the particulate material in transit along the water column.

Taken together, these data indicate that the substances dissolved within the WAF only made the water toxic at very high concentrations. It is very important to note, however, that the FMB-BD7 formulation had very little toxicity even when BW-PM was used. The results with quartz sand confirmed the increase of the particulate effect per se but, without taking account of forecasted environmental fallouts and the subsequent distribution of PM in large volumes of water, these results seem to be overestimated with respect to the potential exposure of natural environment in real conditions.

Data on the impact of the brake particles on edaphic organisms clearly indicate that the WAF solutions did not affect the germination of

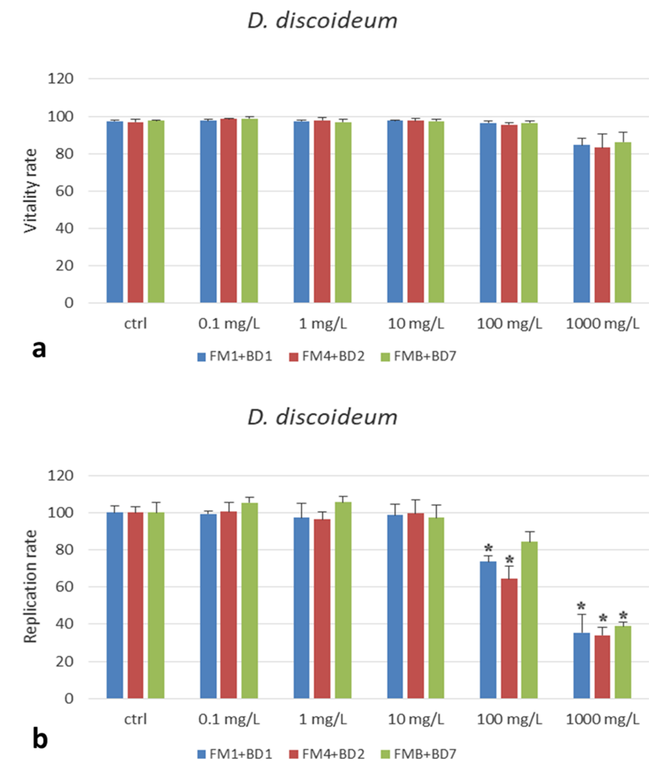

Fig. 7. (a-b). Survival rate (a) and reproduction rate (b) of *D. discoideum* amoebae after exposure to the three brake-system particulate. Data represent the mean ± SD of at least four replicates: *statistically significant differences with respect to control values ($p < 0.05$ Mann-Whitney *U* test).

Table 2
Average PM_{10}dry depositions (in mg $m^{-2}year^{-1}$) associated to FM1-BD1, FM4-BD2 and FMB-BD7 in an extra urban scenario Original daily results for the cold and warm scenario were averaged to provide a single value closer to an annual estimate at different distances from the emission source (i.e. motorway).
EXTRA-URBAN PM_{10}DEPOSITION.

AVERAGE PM_{10}DRYDEPOSITIONS (mg m-2 yr-1)			
brake solution	roadside	200 m	500 m
FM1-BD1	18.65	2.95	0.80
FM4-BD2	17.60	2.64	0.73
FMB-BD7	27.95	4.05	1.13

mono- and dicotyledonous seeds. Instead, the effects of the BW-PM added in a quartz sand substrate were more evident at the highest concentrations only for FM1-BD1 and FM4-BD2. In this case, there is no univocal explanation of the results. The absence of effects on the WAF-exposed seeds seem to exclude that the phytotoxicity depends exclusively on the particle chemistry; this would be confirmed by perturbation in the germination rate in the presence of BW-PM.

The sensitivity of plant models to large quantities of heavy metals (Arambašić et al., 1995; APAT, 2004; Lin and Xing, 2007; Lee et al., 2008; Cuske et al., 2017) such as Cu and Zn, present in large amounts in the FM1-BD1 and FM4-BD2 formulations, is widely established. Therefore, even in this case, it is possible that the physical interactions of the particle aggregates allowed greater accumulation of chemicals adhering to the seeds. This could have amplified the toxic effects at high concentrations or, alternatively, it could have interfered with the water uptake, a limiting factor for seed germination (Ma et al., 2010). The dampening of these negative effects after the addition of BW-PM to the standard OECD soil, simulating natural conditions, could be related to the presence of organic matter (5% peat).

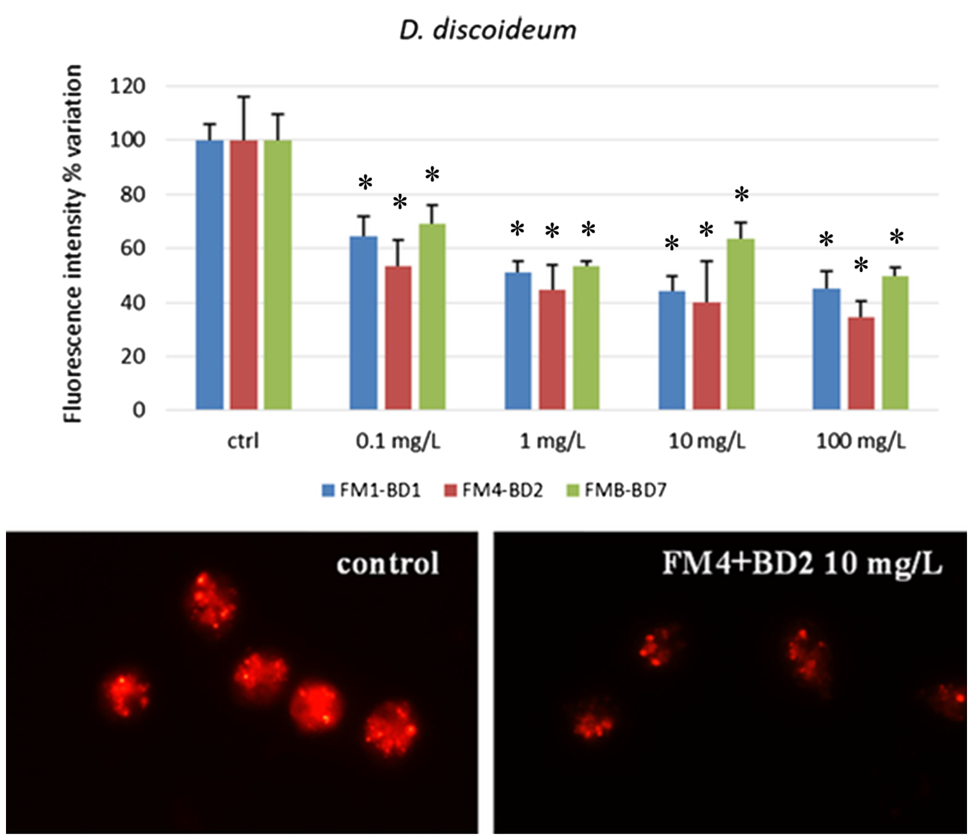

Fig. 8. Lysosomal membrane stability of *D. discoideum* amoebae after exposure to the three brake-system particulate. Data represent the mean ± SD of at least four replicates. Below, representative images of NR-derived fluorescent staining of the lysosomes in cells of control and brake debris-exposed cells (FM4-BD2 10 mg/L).

Neither the nematode *C. elegans* nor the earthworm *E. andrei* were affected by the interaction with the particles of the braking formulations. They are key species for the quality of terrestrial ecosystems (Sochová et al., 2006; Sforzini et al., 2011) due to their quantitative relevance and wide ecological representativeness; therefore, their biological responses to potential contaminant exposure are particularly significant for establishing expected risk levels. These species interact with their environment mainly through the food ingestion but also by direct absorption through the permeable epidermal cuticle, so the entire organism can quickly come into contact with toxic substances dissolved in the interstitial water of the soil (and also of the sediments, in the case of *C. elegans*).

In this context, the link between the eco-toxicological investigations to assess the quality of both edaphic and freshwater environments was represented by the social amoeba *D. discoideum*, which is extremely sensitive to many contaminants starting from sub-cellular levels up to population dynamics (Sforzini et al., 2008). In nature, *D. discoideum* inhabits the pore waters of the soil, where there are concentrations of chemicals and pollutants (Dueri et al., 2008; Gomiero et al., 2012; Magnusson et al., 2013) generally higher than in soils or in waters overlying the sediments (Simpson and Batley, 2016; Sforzini et al., 2016). Since *D. discoideum* is a professional phagocyte, able therefore to quickly and efficiently internalize a great variety of organisms as well as particles (Bozzaro and Eichinger, 2011), it is a particularly significant model organism to investigate the effects of BW-PM. The classical eco-toxicological endpoints (cell viability and growth) did not show up any significant effects, maybe because the amoebic cells are more sensitive to non-essential heavy metals and organic xenobiotic compounds (Sforzini et al., 2016), unrepresentative within the samples tested.

All the results discussed up to this point indicate a very low level of eco-toxicity for the studied BW-PM (especially FMB-BD7) both for soil and freshwater organisms and consequently on the risk for biodiversity.

However, LMS, a very sensitive sublethal biomarker, indicated that all the BW-PM studied significantly lowered this parameter in the amoebic cells, even with the lowest concentration (0.1 mg/L). Activation of the lysosomal vacuolar system is an index of the autophagic activity overload (Sforzini et al., 2018) in response to the recognition of brake wear particles as extraneous bodies. In part this may depend on the physical characteristics of the particulate fraction per se: quartz particles (0.35–3.50 μm) had sublethal effects on *D. discoideum* similar to brake particulate from all the formulations (data not shown).

In addition to low eco-toxicity evidence, FMB-BD7 was ranked as the least emitting brake solution in the urban context, according to the overall dynamometer results. The 3 h-LACT cycle (Los Angeles City Traffic cycle) was chosen, within LOWBRASYS, as a representative reference of as wide use as possible for comparing solutions to reduce particulate emissions from brake wear.

In particular, compared to FM1-BD1, FMB-BD7 had 25% fewer BW-PM_{10} emissions (data not shown). However, predictive data on PM production and subsequent fallout in an extra-urban environment crossed by a motorway gave greater deposition from the FMB-BD7 system (Table 1), reaching and exceeding the values obtained from the other two formulations. For eco-toxicological evaluations in a rural environment we used this more conservative model and found that the risk associated with the release and accumulation of BW-PM is negligible both for aquatic and edaphic ecosystems. Even assuming a 20 cm deep volume of natural soil (in which it is possible to find most of the biomass) near the motorway (i.e. roadside) it would take between 12.165 (for FMB-BD7) and 19.318 (for FM4-BD2) years to reach the maximum concentration tested for *E. andrei*[1]. These values are further overestimated since even in such a conservative scenario, the concentrations tested for the edaphic ecosystem are far higher than realistic environmental concentrations because of the source (brake systems).

It is difficult to apply the same hypotheses for the aquatic ecosystem in order to contextualize the effect concentrations for *P. subcapitata*, *D. magna* and *V. fischeri*: water bodies are extremely dynamic environments and computing realistic concentrations starting from deposition values would call for specific models. Anyway, the order of magnitude of concentrations to which organisms are likely to be exposed is well below those tested.

However, tests on biological models can detect the real ecotoxicity on different endpoints and in this case the in silico approach acts as a support.

In conclusion, the balance between the abatement efficiency in terms of PM_{10} and the lower environmental impacts identifies the innovative FMB-BD7 formulation as the most eco-friendly solution. This does show that the implementation of industrial research contribute to increase the compatibility between new braking system formulations and environmental safety. Evaluation of the eco-toxicological impacts related to the edaphic and aquatic environmental compartments showed that, compared to the commercial FM1-BD1 formulation (already in itself only weakly toxic), the new proposal always had the lowest harmful effects on biological models in acute and chronic bioassays.

In general, compared to the edaphics, aquatic organisms have proved to be more sensitive: while the toxic response to the soluble BW-PM fraction (WAF) was extremely low up to 200 mg/L, the direct mechanical interaction with the particulate gave more pronounced effects, confirmed in parallel by the results of exposure to quartz sand. The effects of an inert substrate on the viability and/or reproduction of aquatic models underlines a focal point: regardless of the origin and chemical composition, the particulate per se has an intrinsic background of toxicity.

The effects detected in edaphic model organisms were minimal, with the exception of the social amoeba *D. discoideum*; this professional phagocyte, due to its greater capacity for particle internalization has undergone more accentuated effects on its reproduction rate but no effects on its viability.

It was interesting to use a model like amoeba to ascertain the appearance of sub-lethal effects. One of the most sensitive early stress biomarkers, LMS, gave significant results with effects already visible at the lowest concentrations. Their early manifestation implies that, beyond any other consideration, it is necessary to take into account that even when present in very small amounts that do not have noxious effects at the organism/population level, the BW-PM may affect the organisms' physiological conditions. The appearance of early sub-lethal effects highlights the importance of considering the particulate a component of environmental contamination that may raise the level of stress, making the organisms more susceptible to other stressors (extreme temperatures and/or droughts and other forms of pollution), aggravating or triggering additional negative effects with substantial impact on biodiversity and environmental health.

CRediT authorship contribution statement

Anna Volta: Investigation, Writing - original draft. **Susanna Sforzini:** Methodology, Investigation, Supervision. **Corrado Camurati:** Investigation. **Federico Teoldi:** Investigation, Formal analysis. **Simone Maiorana:** Investigation. **Alessandro Croce:** Investigation, Resources. **Emilio Benfenati:** Project administration, Writing - review & editing. **Guido Perricone:** Project administration, Resources, Writing - review & editing. **Marco Lodi:** Funding acquisition. **Aldo Viarengo:** Conceptualization, Writing - review & editing, Supervision.

[1] 0.055 (FM1-BD1), 0.052 (FM4-BD2) and 0.082 (FMB-BD7) mg kg-1 year-1 were computed starting from roadside dry deposition values (Tab. 1) assuming a 0.2 m3 soil volume (1 m x 1 m x 20 cm) with 1700 kg m-3 bulk density and no removal and BW-PM accumulation only.

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