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An extensive characterization of various environmentally relevant microplastics – Material properties, leaching and ecotoxicity testing

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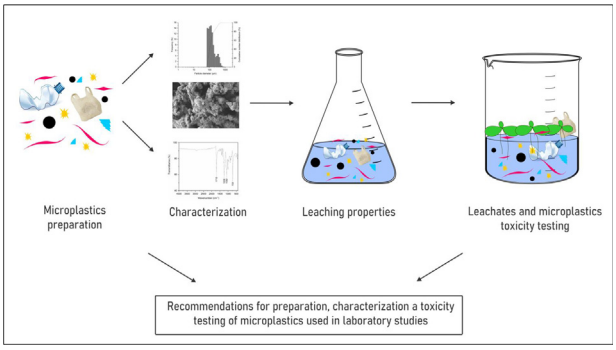
HIGHLIGHTS

- MPs with rough surface and sharp edges had negative impact on duckweed's root length.
- MPs made of Bakelite had negative impact on root length due to leaching of chemicals.
- Natural particles, used as control, showed no negative impact on duckweed.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics in the environment occur in different sizes and shapes and are made of various polymers. Therefore, they also considerably differ in their properties and ecotoxicity. However, the majority of microplastics research uses pre-made spherical microplastics, which practically do not exist in the environment. Our work focused on a comprehensive study of six different types of microplastic that were prepared to simulate common microplastics found in the environment. All types of microplastics where chemically and physically characterized using Fourier-transform infrared spectroscopy, thermal analysis, field-emission scanning electron microscopy, optical microscopy and laser diffraction analysis. The specific surface area was determined using the BET method. Furthermore, effects of microplastics and microplastic leachates on a common duckweed (*Lemna minor*) were evaluated. All tested microplastics did not affect specific growth rate and chlorophyll *a* content in duckweed, while microplastics with a rough surface and sharp edges caused a significant reduction of duckweed root length. Microplastics made of Bakelite also showed an intensive leaching, which increased their ecotoxicity potential. Natural particles used as a control did not have any negative effect on duckweed. Overall, microplastic particles have significantly different ecotoxicity profiles depending on their physico-chemical properties. Therefore, the testing of environmentally relevant particles and their proper characterization, as well as the testing of microplastic leaching properties, is crucial for understanding of microplastics ecotoxicological potential.

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Abbreviations: BET, Brunauer-Emmett-Teller (method); *DSC*, differential scanning calorimetry; *DTG*, first derivative of thermogravimetric curve; *FE-SEM*, field-emission scanning electron microscopy; *FTIR*, Fourier-transform infrared (spectroscopy); *GC*, gas chromatography; *HDPE*, high-density polyethylene; *LDPE*, low-density polyethylene; *MS*, mass spectrometry; OIT_{temp}, oxidation induction temperature;; *PE*, polyethylene; *PET*, polyethylene terephthalate; *PS*, polystyrene; *TA*, thermal analysis; $T_{dec\ from\ DTG}$, the temperature at which the substance chemically decomposes obtained from DTG curve; *TGA*, thermogravimetric analysis; T_m, melting temperature; *XRD*, X-ray diffraction.

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1. Introduction

Over the last decade, research into microplastics has received increased attention due to the extensive occurrence of microplastics in the environment. They have been reported everywhere; in the open ocean (Wang et al., 2020a), coastal areas (Llorca et al., 2020), in various freshwaters (Stanton et al., 2020), wetlands (Ziajahromi et al., 2020), soils (Guo et al., 2020), air (Gasperi et al., 2018), and in various organisms regardless of abundance, trophic level or feeding strategy (Rezania et al., 2018). The majority of microplastic pollution comes from land and is carried by rivers, reaching the marine environment (Rochman, 2018). The most important source of microplastics is the fragmentation of plastic litter already disposed in the aquatic ecosystems (SAPEA, 2019). Another large source is the abrasion of plastic coatings (Horton et al., 2017) and car tires (Kole et al., 2017). Recently, wastewaters have also been identified to carry a significant amount of microplastics (Enfrin et al., 2019). They contain synthetic textile fibres generated during laundry, polyethylene microbeads washed from cosmetics and disintegrated parts of larger consumer products that are flushed down the toilet (Mourgkogiannis et al., 2018; Murphy et al., 2016; Prata, 2018).

Consequently, microplastics in the environment exist in various sizes, and the majority of microplastics found in the environment have irregular shapes: they often exist in the form of fragments, fibres or films (as can be seen e.g. in Goss et al. (2018), Laglbauer et al. (2014) or Nan et al. (2020)). They are made of number of polymers and can contain many additives that are added during plastic production, and their leaching can significantly alter their ecotoxicological profile (Schrank et al., 2019). They also differ in terms of the degree of degradation, associated microorganisms, and the number and concentrations of pollutants that are adsorbed from their surroundings onto their surface (Acosta-Coley et al., 2019; Kalčíková et al., 2020; Liu et al., 2019). All these factors affect the behaviour, fate, and ecotoxicity of microplastics (Kalčíková et al., 2020).

Although the variability of microplastics in the environment is countless, laboratory-based microplastic research have rarely considered these variations in microplastics. The most often used particles in microplastics research are spheres; these are industrially produced, designed to obtain a particle size with desired properties, and are made of a pure polymer, with the most common being polyethylene (PE) or polystyrene (PS) (e.g., in Webb et al. (2020), Wang et al. (2020b) and elsewhere). Although such particles represent microplastics by definition and are easy to obtain, describe, and use, they are rarely found in the environment (Wong et al., 2020). In this context, the aim of our study was to prepare various microplastics that are environmentally relevant and to use several methods for their extensive description and characterization in terms of their size, structure, material, ecotoxicity, and leaching property. We also used natural particles to differentiate the effect of microplastics from the natural background (e.g. Ogonowski et al. (2016); Scherer et al. (2020); Schür et al. (2020)). To our knowledge, this is the most extensive study to date aiming at the preparation and characterization of plastic particles used in microplastics laboratory research with high environmental relevance.

2. Materials and methods

2.1. Microplastics preparation

Six types of environmentally relevant plastic particles in forms of fragments, films and fibres were prepared in this study. They were all labelled as "microplastics" (from A to F) regardless their actual size distribution further determined by particle size analysis (Section 2.2.2). Type A microplastics were extracted from a facial scrub product purchased in an international store; these microplastics are also called microbeads and they were made of PE which was listed among ingredients of the cosmetic product. They were extracted by using the technique described by Kalčíková et al. (2017). Briefly, the facial scrub was dissolved in warm, deionised water, filtered through filter paper (pore size 4–12 μm), washed, and dried in a laboratory dryer. Microplastics B were fibres obtained by the grinding of synthetic polyester clothing by IKA Tube Mill 100 control (6 min at 8000 rpm). Microplastics C were obtained by the grinding of shopping bag (polymer type was not labelled). Microplastics D were industrial microplastics made of Bakelite. These microplastics, in the form of fine powder, were obtained from a local producer of plastics, and this powder is a semi-product that is further used to produce plastic items. Microplastics E were prepared from a common, hard PET bottle which was cut into small pieces. Microplastics F were tyre wear particles obtained from a local car repair service. They originated from an old, used tyre, and we separated larger particles using laboratory test sieves to obtain particles smaller than 500 μm. Except for microplastics, we also tested the properties, ecotoxicity, and leaching of natural particles. As model natural particles, beech (*Fagus* sp) sawdust was selected. These particles were prepared by drilling 10 mm holes in a beech wood slab. The chips were then separated with standard round sieves with a 125 μm and 800 μm frame diameter to obtain particles with sizes between 125 μm and 800 μm (Kalčíková et al., 2020).

2.2. Microplastic characterization

2.2.1. FTIR and thermal analysis

Fourier-transform infrared (FTIR) spectroscopy was used to obtain information about the polymer types present in microplastics. The infrared spectra of samples were recorded on a PerkinElmer Spectrum Two FT-IR spectrometer equipped with UATR module (Dimond) in the wavenumber range from 4000 cm^{-1} to 400 cm^{-1} (resolution 2 cm^{-1}, 10 scans). Background and ATR correction of the spectra was used.

The thermal and oxidative stability of all microplastics was evaluated in a nitrogen and air atmosphere by two thermal analysis (TA) techniques: Differential Scanning Calorimetry (DSC) employing a Mettler Toledo (CH) DSC 1 analyser and Thermogravimetric analysis (TGA) using a Mettler Toledo (CH) simultaneous TGA/DSC 1. The details of the analysis are given in Supplementary information (Section S1).

2.2.2. Surface analysis

The surface analysis included the observation of shape and colour with the naked eye, optical microscopy, field–emission scanning electron microscopy (FE-SEM), and the determination of the specific surface area. The morphologies of microplastics were observed with an optical microscope Zeiss Imager.Z2m (in transmitted light mode) and FE-SEM Zeiss ULTRA plus. All microplastic samples were coated with a thin Au/Pd layer (approximately 10 nm) before FE-SEM analysis. Microscopy was performed at an accelerating voltage of 2 kV using an Evernhart–Thornley detector. The specific surface area of microplastics was determined by Micrometritics ASAP 2020, using the Brunauer–Emmett–Teller (BET) method. The final temperature of the degas was 50 °C and the final vacuum value was 50 μmHg.

2.2.3. Particle size distribution

It is possible to measure the particle size distribution by volume or by number. Since the number of particles (especially small particles) is crucial in microplastics research, we used only the number size distribution in this study. The particle size distribution of microplastics A, B, D, E, F, and natural particles was determined by the laser diffraction analyser Microtrac S3500 Bluewave, where the measurement was repeated three times using the dry unit. The particle size distribution of microplastics C that were in form of thin film could not be determined by the laser diffraction analyser due to difficulties during passage of these microfilms into the initiation chamber. Therefore, particle size measurement was performed with image analysis, where microplastics were placed on a black background with a length-scale and photographed with a digital camera. Pictures were later processed

with the program AxioVision v4.8.2. To obtain statistically reliable data, at least 500 particles were included in the analysis.

The mean number of particles per mg was also determined. For microplastics A, C, D, E, F, and natural particles the specific amount was weighed, photographed, and processed with the program AxioVision v4.8.2, where at least 500 particles were included in the analysis. The mean number of microplastics B (that were in form of fibres) per mg was determined by the separation of numerous fibres. Lengths and diameters were measured with an optical microscope, and the mass of each particle was calculated based on the polymer density (Hidalgo-Ruz et al., 2012). From the mass of each particle, the number of particles per mg was calculated.

2.3. Ecotoxicity

2.3.1. Microplastic particles

The ecotoxicity of microplastics was assessed by the standardized OECD test No. 221: *Lemna* sp. Growth Inhibition Test (OECD, 2006). The duckweed *Lemna minor* used in this study was previously cultivated for several years under laboratory conditions. Experiments followed the standard procedure and were conducted in 100 mL breakers, where the volume of Steinberg medium (OECD, 2006) was 50 mL. The concentration of microplastics and natural particles in experiments was 100 mg/L (equal to $9.6 \cdot 10^3$, $5.8 \cdot 10^4$, $1.1 \cdot 10^2$, $1.5 \cdot 10^8$, $3.5 \cdot 10^2$, $4.5 \cdot 10^4$ and $3.2 \cdot 10^7$ particles/L for microplastics A, B, C, D, E, F, and natural particles, respectively (Table 3)). This concentration was used in our previous studies where microplastics showed an effect on duckweed (Jemec Kokalj et al., 2019; Kalčíková et al., 2020, 2017), but this concentration was also used in other studies (e.g., Besseling et al. (2014)). Prior to the tests, the roots of the plant were removed, and then 10 fronds were added to each breaker. Experiments lasted 168 h in a climate chamber at 24 ± 2 °C, at a humidity of >70% to minimize the evaporation of the media and a ratio of light/dark was 16 h/8 h, with a light intensity of 7000 ± 500 lx. All tests, including the control (without any particles), were replicated six times.

2.3.2. Microplastic leachates

Leaching tests were performed with the same concentration of microplastics—100 mg/L. Microplastics and natural particles without a plant were incubated in 60 mL Steinberg medium for 168 h in at a temperature of 24 ± 2 °C. Leachate was then filtered through filter paper (pore size 4–12 μm). Ten fronds were added to 50 mL leachate and further incubated for 168 h under the same conditions as in tests with microplastic particles (described above). All tests, including the control, were replicated six times.

2.3.3. Ecotoxicity evaluation

After 168 h of exposure, the impact of microplastics and their leachates on *Lemna minor* was evaluated by the determination of the specific growth rate, root length, and chlorophyll *a* content. The specific growth rate was calculated from the number of fronds prior to and after the exposure using Eq. (1):

$$\mu = \frac{ln\,(n_t) - ln\,(n_0)}{t} \quad (1)$$

where μ is specific growth rate (day^{-1}), n_t is number of fronds after exposure (/), n_0 is number of fronds prior to exposure (/) and t is the incubation time (day).

After counting the number of fronds, 10 plants were randomly selected, and their root length was then measured using millimetre paper. Chlorophyll *a* content was determined according to Radić and Pevalek-Kozlina (2010) with some minor modifications, as described in Kalčíková et al. (2016). Approximately 20 mg of fresh plant was homogenized in the dark, using 95% (*v*/v) ethanol. Samples were then stored at a temperature of −18 ± 2 °C for 24 h. The absorbance of the supernatant was read at 662.4 nm and 648.6 nm (Cary 50 UV-VIS, Varian). Chlorophyll *a* contents were calculated according to Lichtenthaler (1987).

The statistical significances of the differences between the control and exposed groups were assessed by the Mann–Whitney *U* test; differences were considered significant if $p < 0.01$ using OriginPro 2020b software (OriginLab Corp. Northampton, MA, USA).

3. Results

3.1. FTIR and thermal analysis

The composition of the samples was derived from FTIR data. As can be seen from Fig. S2, the FTIR spectra of microplastics A and C were very similar, as were the FTIR spectra of microplastics B and E. The strong absorption bands near 2915 cm^{-1} and 2848 cm^{-1} (C—H asymmetric and symmetric stretching vibrations), 1465 cm^{-1} (CH_2 bending), and 718 cm^{-1} (CH_2 rocking) were observed in the spectra of microplastics A and C. Comparison of the spectra with the spectral database confirmed the presence of polyethylene (PE) (John Wiley and Sons, 2020a, 2020b). The magnification of the spectral region from 1400 cm^{-1} to 1330 cm^{-1} of both spectra revealed the difference in the presence of the band at 1377 cm^{-1} (CH_3 bending vibrations) and its intensity relative to 1368 cm^{-1}, indicating that microplastics A were likely to be low-density polyethylene (LDPE) and microplastics C were likely to be high-density polyethylene (HDPE) (Jung et al., 2018).

In the FTIR spectra of microplastics B and E (Fig. S2), the strong absorption bands at 1713 cm^{-1} (C═O stretching), 1238 cm^{-1} and 1092 cm^{-1} (asymmetric and symmetric C—O stretching) and 723 cm^{-1} (aromatic CH out-of-plane bending) were observed (Jung et al., 2018). Both IR spectra match polyethylene terephthalate (PET) reference spectra in the spectral database (John Wiley and Sons, 2020c).

Microplastics D were a semi-product Bakelite (John Wiley and Sons, 2020d), characterized by a broad absorption band at 3270 cm^{-1} (O—H stretching vibrations of phenol) and bands at 2921 cm^{-1} and 2871 cm^{-1} (in-phase and out-of-phase stretching vibrations of alkane —CH_2), 1653 cm^{-1} and 1507 cm^{-1} (C═C stretching in aromatic ring), 1457 cm^{-1} (C—H aliphatic/C—H deformation), 1369 cm^{-1} (C—H deformation), 1236 cm^{-1} (C—O stretching), 1005 cm^{-1} (C—O stretching of CH_2—OH), 810 cm^{-1} (C—H deformations), and 671 cm^{-1} (C—H bending) observed in the FTIR spectrum of microplastics D in Fig. S2 (Aneesh Kumar et al., 2018).

The FTIR spectra of microplastics F (Fig. S2) were comparable to the FTIR spectra of ground tyre rubber (Karabork et al., 2014). The sharp bands at 2915 cm^{-1} and 2847 cm^{-1} could be identified as asymmetric and symmetric C—H stretching vibrations, respectively, and the bands at 1431 cm^{-1}, 1371 cm^{-1}, and 694 cm^{-1} showed a C—H deformation vibration, typical for rubber (John Wiley and Sons, 2020). The strong broad absorption at 1026 cm^{-1} could be assigned to the Si—O stretching vibrations of SiO_2, which is used as a reinforcing filler in compounds for tyres, while the strong broad band near 3290 cm^{-1} was associated with stretching vibrations of the O—H bond.

For dynamic DSC measurements in a nitrogen (Fig. 1, lower graph), all microplastics apart from an amorphous D and F showed a broad melting range with a concave low-temperature side, which is typical for polymers and especially for semicrystalline polymers. The same melting point temperatures (T_m) were also confirmed in an air atmosphere (Fig. 1, upper graph and Table 1), as melting is independent of the atmosphere. The melting points of microplastics A and C were typical for the different PE qualities—102 °C for LDPE and 128 °C for HDPE—as already suggested by FTIR measurements. The proximity of the melting points of microplastics B and E near 250 °C indicated that they belonged to the same polyester family of PET.

After melting, all samples decomposed (Fig. 1, upper graph). The temperature when the oxidation–decomposition process began

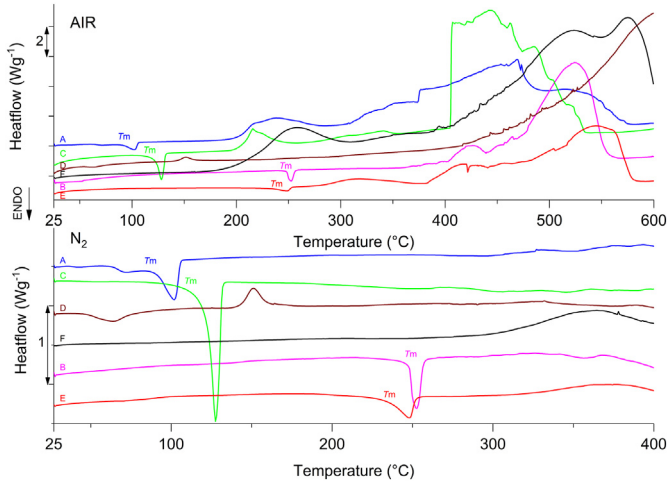

Fig. 1. Differential scanning calorimetry (DSC) curves of microplastics A–F measured in nitrogen and displayed up to 400 °C (lower graph). The upper graph shows the DSC curves in air up to 600 °C.

(OIT_{temp}, Table 1), could be considered as the onset temperature of the exothermic peak.

From the obtained TGA curves in nitrogen, and using its first derivative curves (DTG), we saw that two pairs of microplastics—A and C and, in particular, B and E—had a similar one-step thermal decomposition course (Fig. S3) with a typical pyrolysis region between 340 °C and 550 °C. Microplastics C were slightly more thermally stable, with the decomposition region being shifted to a higher temperature for about 10 °C compared to microplastics A. Unlike A and C, which almost decomposed completely, both PET microplastics B and E contained 16.6% and 11.8% of carbon black residues (Doğan-Sağlamtimur et al., 2019), respectively. The presence of oxygen accelerates and completes the decomposition process in microplastics A, B, C and E (the data obtained from the measured TGA curves in air not shown here are in Table 2).

The TGA measurement of microplastics D in both atmospheres (Fig. S3) showed a mass loss occurring immediately after heating started, which is represented by both DSC curves as an endothermic peak at 64 °C and an exothermic peak at 151 °C (Fig. 1). In air, the DTG curve confirmed the OIT_{temp} value at 432 °C, as it indicated the beginning of the complete combustion of microplastics D up to 600 °C, while in nitrogen, the decomposition process was not yet completed even at 800 °C, as the final carbon yield was greater than 50%, as already reported (Smorygo et al., 2016).

In the case of microplastics F, in a nitrogen atmosphere, the mass stabilized at 500 °C after two major, well-separated decomposition steps with DTG peaks at 366 °C and 444 °C, corresponding to different types of rubber degradation (natural rubber, NR, and styrene butadiene, SBR) with 35.6% of carbon black as residue (Fig. S3). The final combustion in air accompanied by two exothermic peaks began at 460 °C and was finalised at 616 °C with 11.2% residue, which corresponded to ashes from elastomer–rubber, fillers, or glass fibres (Datta et al., 2017; Riesen and Schawe, 2002; Wagner, 2009).

Table 1
Melting point temperatures and OIT_{temp}.

Sample	The composition of microplastics by FTIR	T_m (°C)	T_m (°C)/standard (Riesen and Schawe, 2002)	OIT_{temp} (°C)/AIR
A	PE	102	110	201
B	PET	252	250–255	361
C	PE	128	131–135	202
D	Bakelite	/	/	432
E	PET	248	248	284
F	Tyre rubber	/	/	204

Table 2
Thermogravimetric analysis (TGA) data for all samples obtained under a nitrogen and an air atmosphere.

Sample	Nitrogen atmosphere			Air atmosphere		
	$T_{dec\ from\ DTG}$ (°C)	T_{End} (°C)	Residue at T_{End} (%)	$T_{dec\ from\ DTG}$ (°C)	T_{End} (°C)	Residue at T_{End} (%)
A	359	504	0.2	212	550	0.7
B	339	501	16.6	311	537	0.9
C	360	509	1.0	215	517	1.3
D	77	>800	51.4	79	600	0.8
E	347	533	11.8	273	569	0.4
F	181	502	35.6	180	616	11.2

3.2. Surface analysis

Microplastics A, extracted from a facial scrub, were in the form of a white powder and irregularly shaped (Figs. 2A and 3A). B microplastics were synthetic fibres of a pink colour (Fig. 2B), exhibiting a smooth surface with some wrinkles (Fig. 3B). Microplastics C were film fragments of a transparent shopping bag (Fig. 2C), with a similar surface to microplastics A (Fig. 3C), which could be expected since they are both made of PE (Section 3.1.). Microplastics D were an industrial Bakelite in the form of an orange powder with a number of irregularities on the particle surfaces (Figs. 2D and 3D). Microplastics E were small, colourless fragments of a PET plastic bottle with a relatively smooth surface (Figs. 2E and 3E). Microplastics F, prepared from an old tyre, were the most irregular particles, with a very heterogenous surface (Figs. 2F and 3F). The selected natural particles—beech saw dust—used in this study had a similar shape and surface to microplastics A and F (Fig. S4).

The specific surface areas of microplastics are presented in Table 3. All microplastic particles had relatively small specific surface areas, ranging from 34 cm^2/g to 514 cm^2/g. The specific surface area of the microplastics E was not measurable.

3.3. Particle size distribution

Fig. 4 represents the number particle size distribution of tested microplastics. The mean particle diameter of microplastics ranged from 7.64 ± 3.48 μm to 652.2 ± 47.6 μm (Table 3). Natural particles were in the same size range (133.2 ± 77.6 μm) (Fig. S5). All tested microplastics were polydisperse systems with a wide particle size distribution (Fig. 4), as expected for microplastics that are generated in the environment. Mean length and diameter of fibres (microplastics B), measured with an optical microscope, was 5362 ± 1082 μm and 7.9 ± 0.7 μm, respectively.

The mean number of particles per mg of microplastics varied from 1.1 particle/mg of microplastics to $1.5 \cdot 10^6$ particles/mg of microplastics (Table 3), depending on their size. The mean number of natural particles per mg was $3.2 \cdot 10^5$ particles/mg.

3.4. Ecotoxicity testing

3.4.1. Effects of microplastic particles

All tested microplastic particles and natural particles did not show negative effects on the specific growth rate of duckweed. The average specific growth rates of duckweed in the control condition, with microplastics, and with natural particles were 0.319 ± 0.016 day^{-1}, 0.316 ± 0.021 day^{-1}, and 0.319 ± 0.026 day^{-1}, respectively (Fig. S6a). Moreover, photosynthetic pigment chlorophyll *a* was also not negatively affected by the microplastic particles. Duckweed in control conditions contained 0.53 ± 0.04 mg/g of chlorophyll *a*, while duckweed in the tests with microplastics and with natural particles

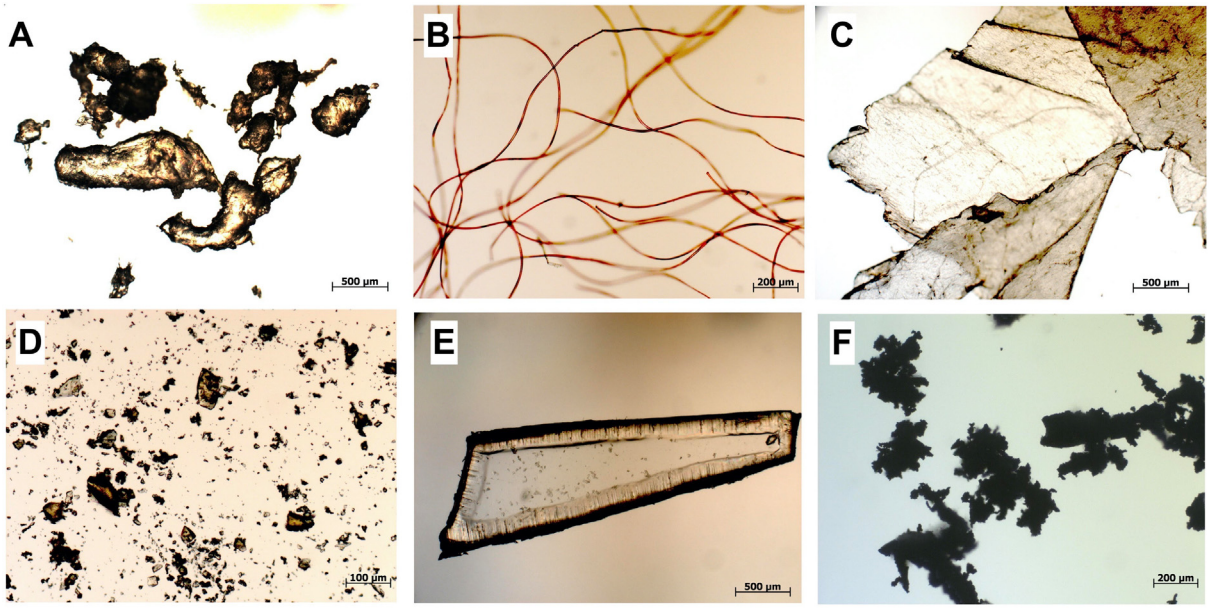

Fig. 2. Optical microscope images of microplastics A–F.

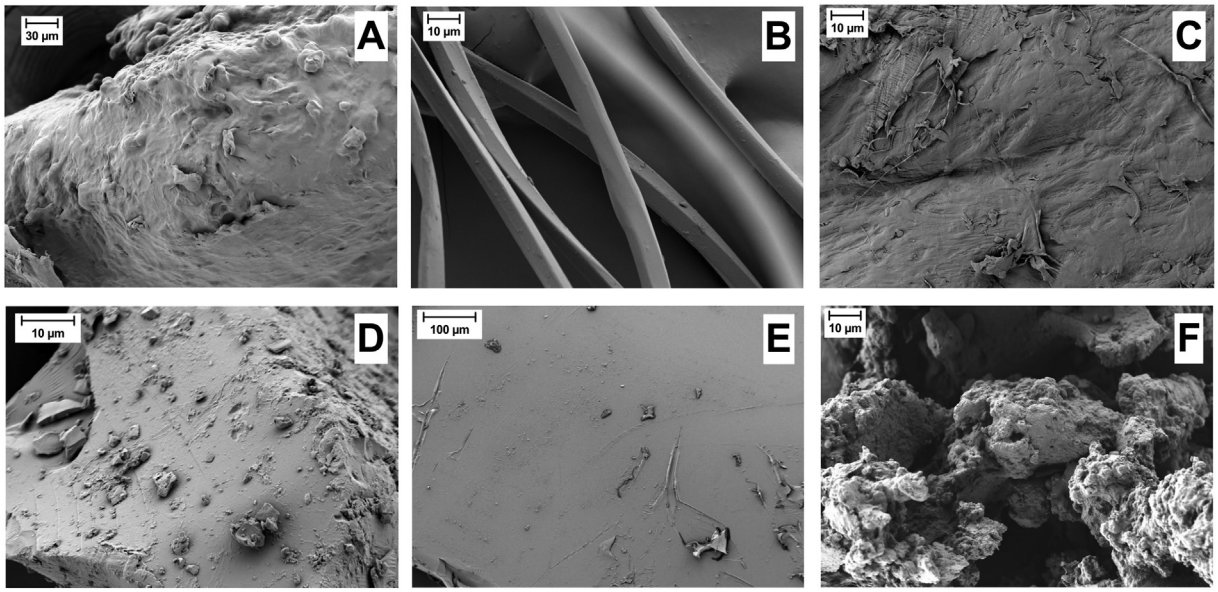

Fig. 3. FE-SEM images of microplastics A–F.

contained 0.50 ± 0.06 mg/g and 0.52 ± 0.07 mg/g of chlorophyll *a* on average, respectively (Fig. S7a).

In contrast, root length was the only significantly reduced of all factors when duckweed was exposed to microplastics A, D and F (Fig. 5a). Type A microplastics reduced the root length of duckweed by 17 ± 7%. However, the most negative effect on root length was shown by microplastics D and F, reducing its length by 40 ± 6% and 37 ± 6%, respectively.

3.4.2. Effects of microplastic leachates

In the next experiment, we prepared leachates from tested microplastics and natural particles and exposed duckweed under the

Table 3
Properties of microplastics A–F: specific surface area, mean number of particles per mg of microplastics, and mean value of number particle size distribution.

Microplastics	A	B	C	D	E	F
Specific surface area (cm^2/g)	62	244	514	249	<1	34
Mean number of particles per mg of microplastics[a]	96	581	1.1	$1.5 \cdot 10^6$	3.5	445
Mean value of number particle size distribution (μm) (mean ± SD, $n = 3$)	148.7 ± 75.4	9.58 ± 3.51	652.2 ± 47.6	7.64 ± 3.48	211.8 ± 51.7	47.39 ± 22.2

[a] Calculated value.

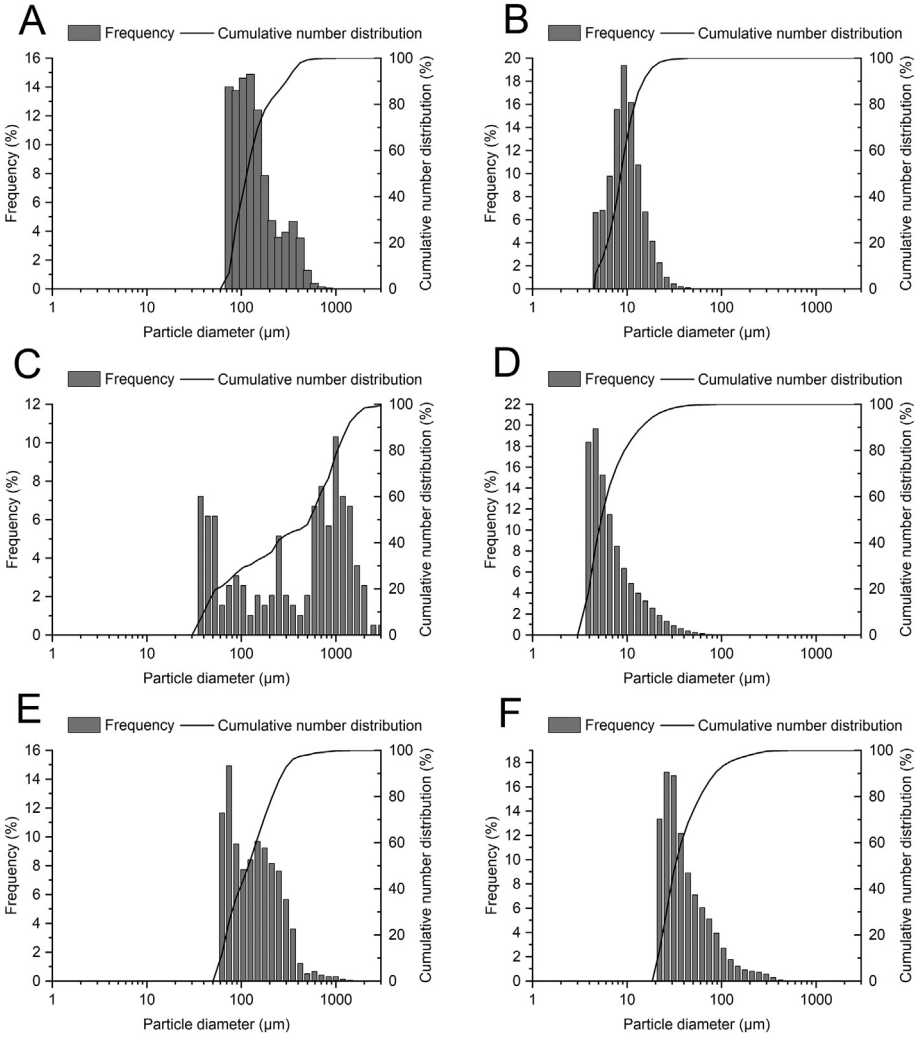

Fig. 4. Number particle size distribution for microplastics A, B, D, E, and F with laser diffraction analyser and C with an image analysis.

same conditions as in the previous test. The specific growth rate of duckweed was not affected when leachates were used as a medium. The growth rate of duckweed in the control test was 0.333 ± 0.032 day^{-1}, while duckweed in leachates from microplastics and natural particles had average specific growth rates of 0.319 ± 0.018 day^{-1}, and 0.302 ± 0.015 day^{-1}, respectively (Fig. S6b). Similarly, chlorophyll

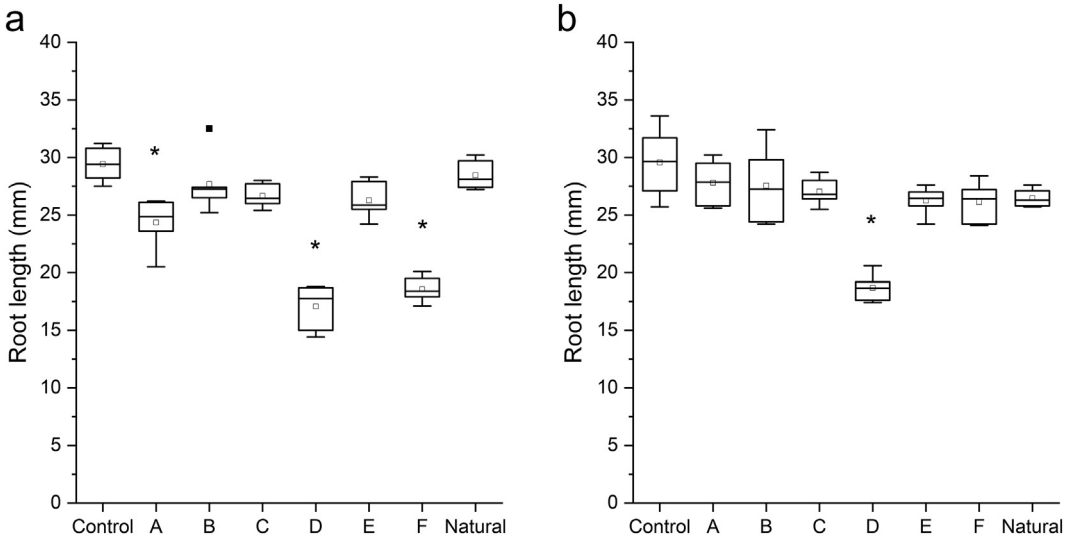

Fig. 5. Root length of duckweed exposed to (a) microplastic particles A–F and natural particles, and (b) microplastic leachates (A–F) and natural particle leachate. * Significant difference in comparison to control ($p < 0.01$), outliers are shown as ■.

a was not negatively affected by leachates of microplastics and natural particles. The average content in control conditions was 0.487 ± 0.022 mg/g, while the average contents in leachates from microplastics and natural particles were 0.488 ± 0.061 mg/g and 0.535 ± 0.048 mg/g, respectively (Fig. S7b). Leachates of microplastics A, B, C, E, F, and natural particles did not have a significant effect on duckweed root length compared to the root length of the control. However, a significant impact on root length was observed when duckweed was exposed to leachate from microplastics D. Comparing its root length to the control, duckweed exposed to leachate of microplastics D had its roots reduced by 31 ± 4% (Fig. 5b).

4. Discussions

The number of research papers dealing with the issues of microplastics has grown exponentially over the last few years (Blettler et al., 2018). Most researchers have used industrially prepared particles, with a spherical shape, uniform size, and with smooth surfaces. However, microplastics found in the environment are not only uniform pellets or spheres, but also different plastic fragments and films from plastic items, such as fishing nets, shopping bags, clothes, etc. (Hidalgo-Ruz et al., 2012). In this context, we prepared, characterized, and tested the ecotoxicity of a variety of microplastics that can be considered environmentally relevant and suitable for microplastic research, because they were prepared in sizes, forms, and from materials that are often released into the environment and consequently found in many ecosystems (Acosta-Coley et al., 2019; Klein et al., 2015).

Chemical composition analysis was performed with FTIR and TA. The non-destructive technique of FTIR spectroscopy is a suitable tool for the determination of particular polymer types in monopolymer plastic samples. In the case of multi-plastic mixtures, the reliable determination of chemical composition by FTIR is quite difficult; thus, the used of additional techniques such as TGA and DSC is needed for appropriate interpretation. For all microplastics used in our study, all techniques provided definite results regarding the composition of the material (typical absorption bands presented, position of melting peaks by DSC analysis, temperature of degradation processes, and residues), and we were able to confirm this on the basis of available standard data.

A rather serious disadvantage of TA techniques in the case of plastics identification is the possibility of signal shifting, since the results of TA analysis are also sensitive to the age of the material, particle size, additives, and impurities. In plastic mixtures, curves and signals can overlap, and thus compositional determination cannot solely depend on one technique. Therefore, complementary techniques such as FTIR or X-ray diffraction (XRD) are required for residues as well as an analysis of evolved gaseous products; for example, the TGA of microplastics with a coupled FTIR, gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) system.

During literature research, we observed that, in many studies, volume size distribution has been used preferably instead of number size distribution. The volume size distribution expresses the percentage of the total volume that each size class occupies in the overall distribution. This means that, in the case of mixed sample with only a few large particles, the volume size distribution will move towards large particles, while many smaller particles (with a comparably small volume) will be overlooked. Since the number of particles—and especially small particles— is crucial in microplastic research, we suggest to use number size distribution over the volume size distribution.

All tested microplastics, except microplastics B (fibres), had irregular shapes. However, it can be seen that all microplastics (including microplastics B) were in shapes (fragments, films, fibres) found in the environment (Acosta-Coley et al., 2019; Klein et al., 2015). When characterizing microplastics by size, Hartmann et al., (2019) suggested that the longest dimension should be considered for their size classification. The mean sizes of studied microplastic particles were less than 1000 μm, however, some particles of microplastics C were also larger than 1000 μm (Fig. 4C). They would be, according to GESAMP (2016), considered as large microplastics (1000–5000 μm). Similar problem showed also microplastics B. They are in form of fibres and the results of the number size distribution analysis done by laser diffraction analyser (Fig. 4B) showed an average mean distribution of 9.58 ± 3.51 μm. However, when lengths of these fibres were measured by an optical microscope, the largest dimension of fibres was 5362 ± 1082 μm. The reason for such difference between these two measurements is that the laser diffraction analyser measures the smaller dimension of the item and therefore the results in case of fibres measurements cannot reflect the actual length that actually define the plastic particle classification (Hartmann et al., 2019).

There has already been a great deal of discussion about the specific surface area of microplastics. It was assumed that the specific surface area of microplastics should be large due to their very good sorption properties and small sizes (Wang et al., 2020c; Wu et al., 2019). However, materials normally exhibit large specific surface areas, mostly due to the large internal surface area which they have due to porosity (Worch, 2012). Polymers are generally not porous, unless they are intentionally produced with high porosity; therefore, in principle, microplastics do not have a large specific surface area, which was already confirmed by Fotopoulou and Karapanagioti (2005), where the specific surface areas of plastic particles found on the beach were measured. FE-SEM images (Fig. 3) showed that none of the tested microplastics had porous surfaces; consequently, specific surface areas ranged from almost immeasurable (microplastics E) to 514 cm^2/g (microplastics C) (Table 3). Our results are in agreement with our previous study, in which various PE cosmetic microplastics had a specific surface area ranging from almost zero to 295 cm^2/g (Kalčíková et al., 2017). Similar findings were obtained also by Hu et al. (2017), where the BET specific surface area was 5320 cm^2/g and 2940 cm^2/g for PE with an average diameter of 56.45 μm and PS with an average diameter of 169 μm, respectively.

In the ecotoxicity test, we used suspensions of relatively high concentrations of microplastics; i.e., 100 mg/L. The mean number of particles per mg of microplastics varied greatly between the tested microplastics (Table 3). Consequently, some of the microplastics concentrations (expressed as particles/L) were environmentally relevant (e.g., microplastics C and E, with concentrations of 110 particles/L and 350 particles/L, respectively) (Pivokonsky et al., 2018); however, some concentrations were significantly higher than concentrations found in the environment (e.g., microplastics D, with a concentration of $1.5 \cdot 10^8$ particles/L). Furthermore, the results of various studies can be compared when calculating the number of particles per mass of microplastics, as some authors performed tests using the concentration given in particles/L and some in mg/L (Prata et al., 2019). Consequently, when studying the impact of microplastics with various densities and sizes, it is more reliable to perform tests with the concentration given in particles/L.

In microplastics research, there are many studies dealing with the assessment of the (eco)toxicity of microplastics. However, only a few of these have focused on both the mechanical and chemical impacts of microplastics. In our study, we first exposed a model organism to microplastics to assess both mechanical and chemical stress (if chemicals are leached from particles). In the second step, we focused on the evaluation of the impact of microplastic leachates (with no particles). Testing microplastic leachates is very important step in understanding whether the impact of microplastics is due to particles or associated chemicals. Many chemicals are deliberately added in order to achieve desired properties (e.g., plasticizers, antioxidants, flame retardants, etc.) (Kowalski et al., 2016; Liu et al., 2020) or are adsorbed from the environment on the surface of microplastics (Jemec Kokalj et al., 2019) and can be leached out during their lifetime (Rani et al., 2017; Sun et al., 2019).

None of the tested microplastics and their leachates affected the specific growth rate of duckweed (Fig. S6). Similar findings have been concluded in previous studies with PE microbeads extracted from facial

scrubs (Jemec Kokalj et al., 2019; Kalčíková et al., 2017), with PS microplastics (van Weert et al., 2019), and with fluorescent red PE microplastics (Mateos-Cárdenas et al., 2019), all of which were obtained from industry. In contrast, Yu et al. (2020) concluded that fluorescent PS microplastics had an impact on a growth of the submerged plant *Utricularia vulgaris*; however, the effect was seen only at the highest concentration of microplastics (140 mg/L).

Microplastics A, D and F most significantly affected the root length of duckweed, but microplastic leachates, except for D microplastics, did not (Fig. 5). This implies that the effects of microplastics A and F were mechanical because of particle shape, whereas the effect of microplastics D was chemical as a result of leaching of chemicals. It has already been suggested (Kalčíková et al., 2017) that microplastic particles can impact root length due to the irregular shape of particles and rough surface, which is in accordance with the surface analysis of the A and F microplastics. This is also supported by results obtained by Mateos-Cárdenas et al. (2019), where smooth PE spheres did not cause negative effects on duckweeds root length. Unlike in some other studies, we could not find a clear connection between the sizes of microplastics and their ecotoxicological effects.

Leachate from the A, B, C, E, and F microplastics did not show any measurable toxic effect. Similar findings were also obtained by Jemec et al. (2016), when GC–MS analysis of microplastics leachates did not detected any leached chemicals. For microplastics A, B, C, and E, this was expected, because these microplastics were prepared from common plastic items that are routinely use for food storage, clothing, and cosmetics, and thus the leaching and toxicity should be low. However, microplastics F were prepared from used car tyres, and different pollutants could have been adsorbed during their usage; e.g., motor oil, cooling liquid, or other road wear particles (Bänsch-Baltruschat et al., 2020). Furthermore, early research into their toxicity indicated that the toxicity of tyres is mainly due to their leachate (Stephensen et al., 2003; Wik and Dave, 2006); therefore, the result that no impacts were seen with microplastic leachate was intriguing. However, similar conclusions were obtained by Khan et al. (2019), where *Hyalella azteca* was exposed to different concentrations of tyre particles and their leachates. When exposing the organisms to tyre particles, concentration–response curves were obtained from which the LC_{50} was derived; however, when testing the leachates, no concentration–response pattern was confirmed (Khan et al., 2019). In general, the most common components of tyres are natural rubber (e.g., polyisoprene), synthetic rubber (e.g., styrene butadiene rubber), filler (typically composed of carbon black, silica and chalk), softener (e.g., oil or resin), vulcanization agents (sulphur and zinc oxide), and other additives, such as preservatives, desiccants, and plasticizers. However, all these chemicals need to be very well cross-linked, as tyres must meet certain standards, including high friction forces, different environmental conditions, and temperature changes (Bänsch-Baltruschat et al., 2020), therefore it is unlikely that these chemicals will leach under the duckweeds' growth conditions. Or it is also plausible that the rubber additives could be partially leached during tyre lifetime and therefore only a low amount of chemicals can be then leached from used tyres.

The leachate of microplastics D was the only leachate to cause a significant decrease of the root length of duckweed (Fig. 5b). Microplastics D were made of Bakelite, which is synthetized from phenol and formaldehyde, and hexamethylenetetramine is usually added as a crosslinking agent (Hocking, 2005). Following the FTIR spectra (Fig. S2), there could be a sign of hexamethylenetetramine being present into microplastics D. Therefore, not knowing the exact concentration of the added chemicals, it is plausible that a phenol and/or crosslinking agent is being leached to the medium, considering that phenol is known to be toxic towards duckweed (Barber et al., 1995). However, additional analysis of this leachate should be performed for more exact conclusions.

Photosynthetic pigments were not negatively affected by microplastic particles and their leachates (Fig. S7). This is in agreement with our previous study (Kalčíková et al., 2017), but a contrasting finding was observed by Yu et al. (2020), where PS microplastics significantly affected the chlorophyll *a* of a submerged aquatic plant, even at the lowest concentration of microplastics (15 mg/L). The authors suggested that chlorophyll *a* is most likely to be reduced due to the adsorption of microplastics on the plant leaves, which overwhelms them and prevents a normal composition of pigments and proteins (Yu et al., 2020). During our experiments with duckweed, the leaves were not shaded by microplastics, as the microplastics floated together with the leaves on the medium surface; thus, there was no noticeable effect on chlorophyll *a*.

Alongside all ecotoxicity tests, we also tested natural particles to determine whether they affected duckweed. Although they were made in sizes and shapes similar to microplastics A and F that showed significant impacts on root lengths, natural particles did not affect duckweed at all. We assume that the difference can be explained by the material hardness. Natural particles did not cause any measurable effect because they are made of soft material (wood), while the hardness of plastics could be responsible for their mechanical stress on duckweed.

5. Conclusions

In summary, microplastics have various physico-chemical properties that affect their ecotoxicity. Our study showed that those with rough surfaces and particles with sharp edges had a higher ecotoxicity potential, while microplastics with smooth surfaces posed a comparable effect to natural particles. The particles' morphology seems to be more important than the particles' sizes when evaluating the impact of microplastics on duckweed. Some microplastics (in our study, observed with Bakelite) can also pose a chemical stress by leaching additives or unreacted chemicals used during polymerization into the surrounding environment. Currently, microplastics represent one of the most complicated environmental issues, because the evaluation of their environmental impact is extremely complex. Therefore, we suggest using a variety of particles in microplastics research considering their environmental relevance in order to characterize them in terms of their size, shape, material, and surface morphology; it is also necessary to compare their effect to a control—natural particles—and also to assess their leaching properties. We also believe that vascular plants, which so far have been largely avoided in microplastics research, should be used in ecotoxicity evaluation more often, because they are also a crucial part of natural ecosystems and in some cases are more sensitive to microplastics than animal species.

CRediT authorship contribution statement

Ula Rozman: Investigation, Conceptualization, Data curation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Tilen Turk:** Investigation, Data curation, Visualization, Writing – original draft. **Tina Skalar:** Investigation, Methodology, Validation, Formal analysis. **Marija Zupančič:** Methodology, Validation, Writing – original draft. **Nataša Čelan Korošin:** Methodology, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Marjan Marinšek:** Methodology, Validation, Writing – original draft. **Jesus Olivero-Verbel:** Conceptualization, Writing – original draft. **Gabriela Kalčíková:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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