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Diversifying endpoints in biodegradation testing of microplastics

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Abstract

To counteract microplastic (MP) pollution the European Commission adopted a restriction of intentionally adding synthetic polymer microparticles to products, such as detergents, rinse-off cosmetics, controlled-release fertilizers or pesticides. Exempted are particles consisting of polymers that, e.g., meet the (bio)degradability pass criteria of the available test methods. The main criterion for proving biodegradability is the particle's mineralization rate, as set out, amongst others, in OECD testing guidelines 301B referenced by the REACH regulation of the European Union. Since present test methods are designed and validated to test low-molecular, soluble compounds adaptations regarding MP biodegradability testing are of high interest. In this study, the biodegradability of a polyurea (PUA) microcapsule suspension was tested using a standard degradation test method (OECD test guideline (TG) 301B). Since the polymeric component comprised less than 1% of the suspension, besides the aromatic solvent inside the microcapsule (8.6%) and water (90.9%), ^{14}C-labeling of the polymer was essential for specific detection throughout the experiments. Particle size determination of the tested PUA microcapsules indicated a bias in the test results due to the presence of a soluble ^{14}C-compound, a byproduct of synthesis, identified using ultra-high performance liquid chromatography–high resolution mass spectrometry (UHPLC–HRMS) coupled with radioactivity detection. This study highlights the need for proper characterization and purification of the tested particles prior to biodegradation testing and suggests how to diversify future regulatory testing for a comprehensive assessment of the biodegradation of MPs.

Keywords Biodegradation testing, Size fractionation, Radiolabeling, Transformation products, HRMS, Regulatory assessment, Polyurea, Microcapsules, Radioactivity detection, OECD TG 301B

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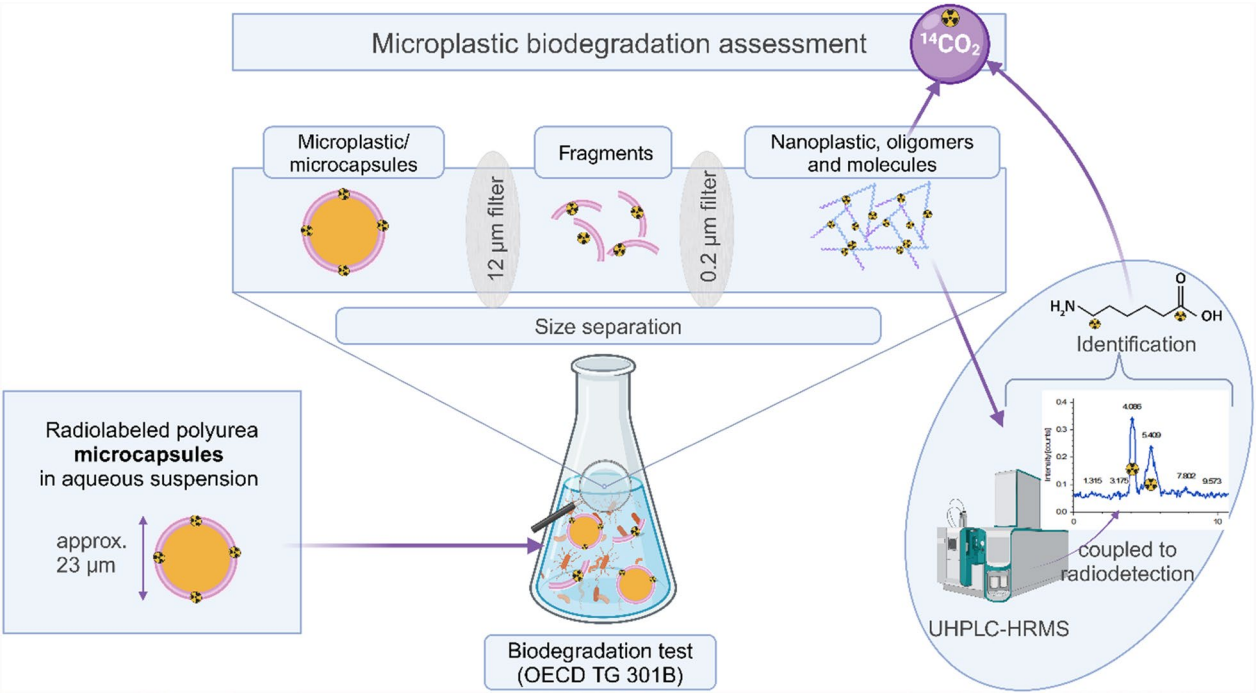
Graphical abstract



Background

The widespread presence of microplastic (MP) pollution, even on mountaintops and down to the deepest oceans, poses substantial challenges to efforts aimed at protecting environmental and human health from related hazards. Strategies to reduce plastic emissions to the environment include the adopted restriction of intentionally added MPs to products under REACH regulation by the European Commission [1]. In accordance with this restriction, MPs are to date defined as solid synthetic polymer particles in a size range of 100 nm to 5 mm. The restriction also includes polymers that are contained in particles constituting at least 1 wt% (percentage of the total weight of the product) or building a continuous surface coating on such particles. Exempted from the restriction are polymers that (i) have a solubility greater than 2 g/L in water, (ii) do not contain carbon atoms in their chemical structure, (iii) are natural and have not been chemically modified, or (iv) for which fulfilment of biodegradability criteria can be demonstrated. In this context, methods to prove biodegradability of MPs are of key relevance. There are overall 13 test methods accepted to prove synthetic polymer microparticle biodegradability, which are organized into 5 groups (screening and simulation test methods) (*Entry 78—Rules on proving degradability*, Appendix 15 of Annex XVII of (EC) No. 1907/2006 [1]) and are displayed in the supplementary information (SI—Table S1).

The present regulatory approach defines the main criterion for biodegradability of MP particles based on their mineralization, which refers to the biological breakdown of the polymer into carbon dioxide (CO_2) and water. Complete mineralization (also referred to as ultimate biodegradation) signifies that the polymer is fully degraded into CO_2 and biomass through microbial metabolism, leaving no residual transformation products. In regulatory testing, biodegradability is often assessed based on the extent of mineralization over a defined timeframe. For instance, reaching ≥ 60% mineralization within a specified test duration of 28–60 days (according to OECD TG 301 B, C, D, F, [2] and OECD TG 310 [3] for Group 1 and 2) is considered sufficient to demonstrate biodegradability.

Tests for MP biodegradability should be performed with particles of a comparable composition, shape, size and surface area as present in the product, if the product meets the "microplastic" definition. However, many products show a high degree of complexity, e.g., due to a wide range of components with different sizes and

particle shapes and/or chemical composition, which might be mineralized to different extents at varying rates. Consequently, the test results could be falsely interpreted.

From a scientific perspective, the biodegradation of polymers is divided into four steps: biodeterioration, depolymerization, bioassimilation and mineralization [4, 5]. During this process, Albright and Chai [6] defined the loss of one key property of the polymer, such as mass fragmentation or tensile strength, as primary degradation. However, a regulatory definition of primary degradation of polymers is still missing. Within the depolymerization the polymer is disintegrated into low-molecular weight compounds. The released organic compounds can be assimilated by microorganisms and, in the last step, either completely decomposed into simple, fully oxidized molecules like carbon dioxide (CO_2), methane, nitrate or ammonium to generate energy or transformed into biomass [5, 7, 8]. However, in addition to mineralization, other critical steps of polymer biodegradation, including biodeterioration, depolymerization, and bioassimilation, receive limited attention in the current regulatory framework for MPs. These intermediate degradation processes may influence the overall breakdown of polymers by altering their molecular structure, reducing particle size, and increasing bioavailability for microbial assimilation. Neglecting these factors in regulatory assessments may lead to incomplete evaluations of polymer degradability and potentially incorrect assumptions of the environmental persistence of MPs and the formation of persistent polymeric fragments.

The permitted test methods to prove biodegradability were designed and validated to test well-defined low molecular weight, mono-constituent and soluble or uniformly dispersed substances. For this type of substance, using the endpoint mineralization to CO_2 or related O_2 consumption is a reasonable and validated approach, however, transferring methods and endpoints to complex particulate polymers is criticized [6, 9–11]. The corresponding ring-trials of most of the standardized tests did not include polymeric particles, hence requiring further testing.

The inclusion of additional types of analysis, like size distribution and related changes of the tested material, can, besides describing or giving a hint for its degradation pathway, help to interpret test results of ultimate degradability (mineralization) and make them more robust and comprehensive. Since size can also play a major role regarding the bioavailability of MPs, characterizing their size distribution is meaningful in this context, too. However, integrating analytical approaches for studying biodegradation pathways is challenging and scarcely investigated.

Polymeric microcapsules used, e.g., for encapsulation of pesticides are also covered by the scope of the EU restriction. However, testing their biodegradation for regulatory purposes raises additional challenges. For example, it is practically impossible to distinguish the mineralization of the microcapsule shell, usually constituting approximately 1 to 5% of the product, from other organic components of the suspension, such as the oil and the pesticide inside the capsule, within the biodegradation test. To comprehensively monitor the biodegradation of synthetic MP capsules we used radioactive isotope-labeling of the polymer, i.e., ^{14}C-labeling, to quantify the $^{14}CO_2$-evolution exclusively as a result of the polymer mineralization, which can substantially enhance sensitivity and specificity.

In this study we investigated the biodegradation of a polyurea (PUA) capsule suspension (CS) used for pesticide applications using OECD TG 301B [2]. For specific tracking of the mineralization of the polymeric shell, the PUA was synthesized using a ^{14}C-labeled monomer ([1,6-^{14}C] hexamethylenediamine). To obtain information about the fragmentation of the capsules, we monitored changes in MP size distribution using sequential filtration. To analyze the presence of soluble compounds either released from the microcapsules or remaining from their synthesis we applied an ultra-high performance liquid chromatography—high resolution mass spectrometer (UHPLC–HR-MS) coupled to a radioactivity ("radio") detector. ^{14}C-labeling further served to examine the source of the detected $^{14}CO_2$ evolution during degradation of the PUA CS. This study aimed to (i) evaluate the biodegradability of PUA CS within the framework of a regulatory approved method and (ii) to assess the potential formation of polymeric fragments or transformation products.

Material and methods

Radioactive labeling and capsule suspension synthesis

The PUA CS was produced in a similar way as described by some of the authors in the WO2021136758 [12] applying the most widely used microcapsule synthesis [13], which is generated by the interfacial polymerization of diisocyanate and polyamine in an emulsion containing an organic-phase and water. For synthesis of radioactively labeled microcapsules, the synthesis procedure of the technical product was carried out on a small scale (factor of approx. 200:1 to production scale; approx. 10:1 to laboratory scale). Hence, a lower concentration and smaller high shear equipment (ULTRA TURRAX Tube Disperser, IKA-Werke GmbH & CO. KG, Germany) had to be used to be able to prepare the initial oil-in-water emulsion for the capsule synthesis. For the procedure, [1,6-^{14}C]hexamethylenediamine (0.08 Wt% of the

undiluted CS) and non-labeled modified polymeric diphenylmethane diisocyanate (pMDI), amounting to 0.24 Wt% of the undiluted CS, were used (3.49–3.57 MBq/mg, Bayer AG, Germany). Since the monomers consist of ≥ 2 binding sites the polymer rather crosslinked generating a polymer structure that is insoluble, more rigid and aging-resistant [14]. Given these properties, substantial biodegradation is not expected for the general polymer. However, for the tested polymer, the pMDI had been modified to include more ester bonds into the polymer structure with the goal of increasing biodegradability. Other components of the suspension included the organic phase, an oil (8.59 Wt% of the undiluted CS, Solvesso 200 ND, ExxonMobil, USA), a dispersant (0.23 Wt% of the undiluted CS, Reax 88, Ingevity Holdings SPRL, USA), and water (in total 90.9 Wt% of the undiluted CS).

The synthesized CS suspension was qualitatively examined by microscopic analysis (SI—Fig. S1) and a quantitative size distribution analysis was performed by laser diffraction using a particle size analyzer (Mastersizer 2000, Malvern, SI—Tables S2, S3 and S4, Fig. S2). In this analysis, the particle size distribution was expressed as a volume-based distribution, representing the percentage of the total sample volume occupied by particles within specific size classes ($\%_{\text{particle-volume-based}}$).

Biodegradability tests

The biodegradation of the aqueous PUA CS was tested in a modified OECD 301B "CO_2-Evolution Test" (OECD TG 301B [2]). In addition to the radiolabeling of the polymer (section "Radioactive labeling and capsule suspension synthesis"), the modifications included (I—section "Preparation of the test inoculum") the use of filtered activated sludge supernatant, (II—section "Biodegradation of the bulk capsule suspension") a prolongation of the test duration, and (III—section "Biodegradation of the different size fractions of the CS") a sequential filtration of the samples for size fractionation. Within the biodegradation tests, the proportion of mineralization was calculated based on the evolved $^{14}CO_2$, which was trapped in sodium hydroxide (NaOH) implementing a flow-through setup as proposed in the OECD TG 301B. The radioactivity within the traps was quantified using liquid scintillation counting (LSC) (section "Quantification and data analysis"). Since the PUA was ^{14}C-labeled, the aeration of the flow-through setup could be done using ambient air instead of CO_2-free synthetic air. The NaOH traps were sampled in intervals of approx. 2 to 27 d during the incubation time, depending on the current $^{14}CO_2$ evolution rate. In all tests the degradability of the standard reference substance, ^{14}C-labeled sodium benzoate (benzoic acid [ring-^{14}C (U)] sodium salt, 130 mCi/mmol, American Radiolabeled Chemicals Inc., USA), was assessed in parallel to monitor the overall degradation performance of the inoculum.

Preparation of the test inoculum

We used filtered activated sludge supernatant instead of the commonly used standard inoculum (mineral medium with activated sludge suspended solids), to reduce the impact of matrix (sludge particles) and to enable the application of as many analytical methods as possible. For this, in a preliminary biodegradation test (OECD TG 301 F [15]), the performance of the filtered activated sludge supernatant on mineralizing sodium benzoate was compared with the mineralization performance of the standard inoculum. For the preparation of the filtered inoculum, activated sewage sludge was collected from the outflow of a wastewater treatment plant (Ruhrverband Kläranlage, Sunthelle 6, 57392 Schmallenberg) 2 d before the start of the experiment and left standing at room temperature in diffuse light for approx. 30 min to let most of the particles settle. The formed supernatant was decanted, centrifuged (5 min at 10000*g*) and filtered (Macherey–Nagel, Mn- 672, Cellulose, average retention capacity 4–12 µm). During the preparation and incubation, overnight aeration of the aqueous inoculum with ambient air was provided.

Biodegradation of the bulk capsule suspension

The total carbon content of the CS was determined using a macro-elemental analyzer (multi EA 4000, Analytik Jena). Both the CS and sodium benzoate test samples were spiked to yield a total carbon (TC) content of approximately 20 mg/L, meeting the requirements of the OECD 301B guideline. At the test start a total of two samples were prepared for sodium benzoate application and 16 samples were prepared with inoculum for PUA CS application (SI—Table S5). All samples and corresponding incubation times are displayed in Fig. 1.

Process controls were implemented to observe whether the degradation performance of the microbes was still functional during the incubation time. For this purpose, ^{14}C-sodium benzoate was applied to an inoculum sample vessel at day 0 and to another inoculum sample vessel at day 14 and the $^{14}CO_2$ evolution over time was monitored. Inoculum samples containing the CS were used for (a) the sequential filtration (section "Biodegradation of the different size fractions of the CS"); (b) the determination the CO_2 bound as carbonic acid in the aqueous media by applying hydrochloric acid (HCl); (c) a toxic control tested in parallel to determine inhibitory effects of the suspension on microbial activity by adding a readily degradable reference compound, here ^{14}C sodium

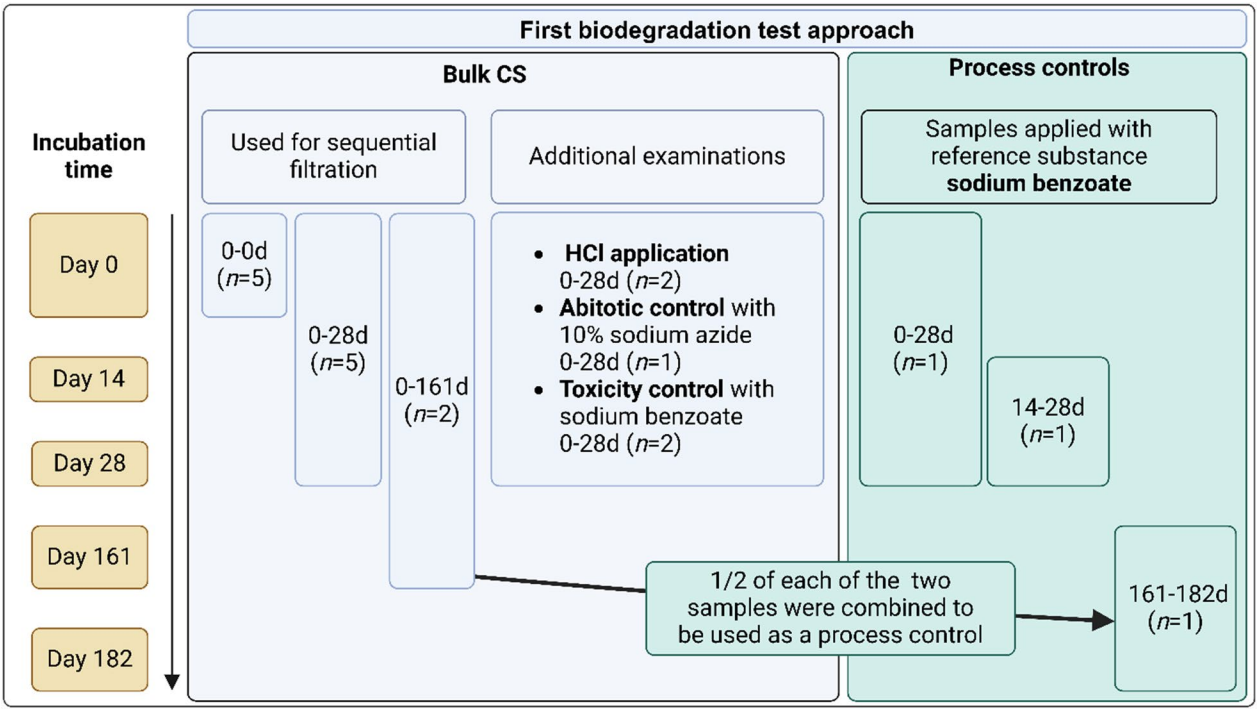


Fig. 1 Overview of samples and their incubation times, submitted to the OECD 301B biodegradability test of the bulk capsule suspension (CS). Samples supplied with CS are displayed with light blue background and process controls, supplied with sodium benzoate are displayed with green background. ½: half of each of the two replicates with bulk CS was sampled at 161 d and the other half combined to a sample to which the standard reference, sodium benzoate, was applied

benzoate (20 mg C/L, if microbial degradation of the reference compound was substantially reduced in the presence of suspension, it would indicate that the suspension has toxic or inhibitory effects on microbial activity); (d) abiotic degradation examined by adding 30 mL of a 10% sodium azide solution. Sodium azide was selected as a bacteriostatic agent because it effectively inhibits microbial respiration and enzymatic activity without altering the chemical structure of microplastic particles or interfering with CO_2 measurements, since it primarily inhibits microorganism growth [16]. Two samples containing CS were incubated for a prolonged test duration (161 d instead of the standard duration of 28 d), to verify that the mineralization potential of the microorganisms had not decreased over this prolonged incubation time. For this, a fraction of the volume of each duplicate was sampled and the remaining fraction of both duplicates was combined to one sample to which the standard reference sodium benzoate was applied, as a last process control starting at 161 d.

The applied radioactivity of each sample was determined after the respective application of the unprocessed stock CS (5 mL with 5.4 ± 0.1 kBq/mL) or of the sodium benzoate stock solution (5 mL with 65.8 ± 0.3 kBq/mL). The radioactivity of the samples was verified at the test start using 1 mL aliquots of each sample ($n = 3$), which were submitted to combustion and LSC determination (section "Quantification and data analysis").

Biodegradation of the different size fractions of the CS

In the first biodegradability test (OECD TG 301B) a sequential filtration was performed to assess changes in the size distribution of the ^{14}C-labeled synthetic polymer microparticles over the incubation time. For this purpose, sample aliquots of 50 mL were sequentially passed through several filters connected in series (12 µm pore size (Cytiva Whatman®, nitrocellulose filter, Z696919), 5 µm pore size (Sartorius, cellulose acetate filter, Type 12,342), 0.45 µm pore size (Sartorius, cellulose acetate filter, Type 11,106) and 0.2 µm pore size (Sartorius, cellulose acetate filter, Type 11,107)) at day 0, day 28 and day 161 (see Fig. 1), and the radioactivity of the filters and the filtrate was quantified.

In a second biodegradability test (OECD TG 301B) the size fractions of the CS were tested separately, by first filtering the CS and testing the size fractions of approx. > 12 µm (filter, Cytiva Whatman®, nitrocellulose filter, 12 µm pore size, Z696919) and approx. < 12 µm (filtrate) separately. For this purpose, 500 mL of the aqueous

stock suspension with a radioactivity of 1.6 ± 0.0 kBq/mL were produced. After thorough shaking, 50 mL CS aliquots were filtered through a 12 µm pore-sized filter ($n = 3$). Each filtrate was sampled for the application to the biodegradation test. The filter was then washed with 100 mL water. Each filtered material and each filtrate were subsequently applied to the inoculum of the biodegradation test and connected to the ventilation system. The same was done with 50-mL aliquots of the unfiltered stock CS ($n = 3$) as a quality control, to verify whether the sum of the mineralization of the fractions on the filter and in the filtrate corresponded to the mineralization of the original 50 mL CS.

Ultra-high performance liquid chromatography with high-resolution mass spectrometry (UHPLC–HR-MS)

The UHPLC–HR-MS analysis coupled with radio detection was used to analyze the smallest filtration fraction (filtrate < 0.2 µm) of the PUA CS and thus, to verify if ^{14}C-labeled molecular compounds remaining from capsule synthesis were present in this fraction. Therefore, UHPLC equipped with a C18 (RP18, ODS, Octadecyl) reversed phase (ODS-AQ, YMC, USA) column, coupled to a Q-Exactive Plus Orbitrap mass spectrometer (HR-MS, using the analysis software Qual Browser of Xcalibur Version 4.0, Thermo Fisher Scientific Inc., USA) and additionally to a radio detector (LB509—YG 75 S6M, Berthold GmbH & Co. KG, Germany) (SI—Tables S6, S7, S8) was employed for qualitative assessment. The LC system was also evaluated in terms of possible adsorption of the analytes to the column matrix. Thus, a recovery test of the radioactivity eluted from the column was performed, by sampling the eluate in time intervals (0–3.5 min; 3.5–7 min; 7–15 min; from 15–60 min in 7.5 min intervals) based on the signal of the radio detector during the whole analytical run. The eluted radioactivity was then determined by LSC (HIDEX 600 SL Counter, HIDEX, Germany). Furthermore, identified molecules within HR-MS analysis were compared to analytical standards of the respective substance. In a next step, we screened for molecules that originated from the labeled [1,6-^{14}C]hexamethylenediamine used for polymer synthesis by performing an isotopic pattern analysis. For this purpose, the isotopic patterns of molecules, present in labeled and non-labeled form, were assessed. In this approach, specific mass shifts of carbon isotopes (e.g., molecules consisting of different compositions of ^{12}C, ^{14}C or $^{14}C_2$, and the specific intensity of ^{12}C-, ^{14}C- or $^{14}C_2$-molecules) were determined. All molecules that eluted with the same mass shift (within 5 ppm) and intensity characteristics (within 20% deviation) were then extracted from the mass spectra. The intensity counts of those molecules were plotted against their retention time (RT). Details of the analysis are given in the supplementary data (SI—Fig. S3).

Quantification and data analysis

In general, the radioactivity of the CS was determined by applying an aliquot of 1 mL in a cellulose cone, leaving it for air-drying overnight followed by combustion in an oxidizer (HIDEX, 600 OX) and quantification of the trapped $^{14}CO_2$ by LSC (HIDEX 600SL). Regarding the quantification of radioactivity remaining on each filter within the sequential filtration, filters were air-dried and combusted. Similarly, the radioactivity of the filtrate was determined by drying 5 × 1 mL aliquots on a cellulose cone followed by combustion. For all remaining radioactive liquid samples or stock solutions, aliquots were directly applied into 4 mL LSC cocktail (Supersolve X, Zinsser Analytics) prior to the LSC determination.

The results are presented as percentage of the recovered radioactivity relative to the applied radioactivity ($\%_{AR}$). For all calculations Microsoft Office Professional Plus Excel (2019) was used. Graphpad Prism 6 was employed for the graphical representation. The graphical abstract and Fig. 1 were created with BioRender.com.

Results and discussion

Biodegradation of the bulk CS

In a pretest, we compared the filtered supernatant and the standard inoculated mineral medium by the biodegradation of the standard reference compound sodium benzoate in an OECD TG 301 F [15] by means of the oxygen demand. In this test, no substantial differences regarding mineralization performance for the reference substance and the related test validity, namely mineralization of > 60% of the reference substance within a 10-d window, were observed (SI—Fig. S4).

Regarding the biodegradation performance of the modified OECD 301B biodegradation test run examining the bulk suspension, 58.6%$_{AR}$ was mineralized by the process control applied on 0 d, approaching the validity threshold (i.e., 60%) set by the TG. Overall, the process controls proved that the microbiome of the filtered supernatant was sufficiently active during the whole incubation time (SI—Fig. S5). The toxicity control showed the combined mineralization of sodium benzoate and the CS (83.6%$_{AR}$; $n = 1$; Fig. 2), revealing that the CS itself did not show toxic or inhibitory effects. In the abiotic control (with sodium azide added), 13.3%$_{AR}$ ($n = 1$) $^{14}CO_2$ was formed over the duration of 28 d with a lag phase of 5 d (Fig. 2) after the microbiome recovered from the sodium azide application from day 7 onwards. Since sodium azide has a bacteriostatic rather than a bactericidal effect [17], a recovered viability of the test inoculum during the incubation time is possible which might delay

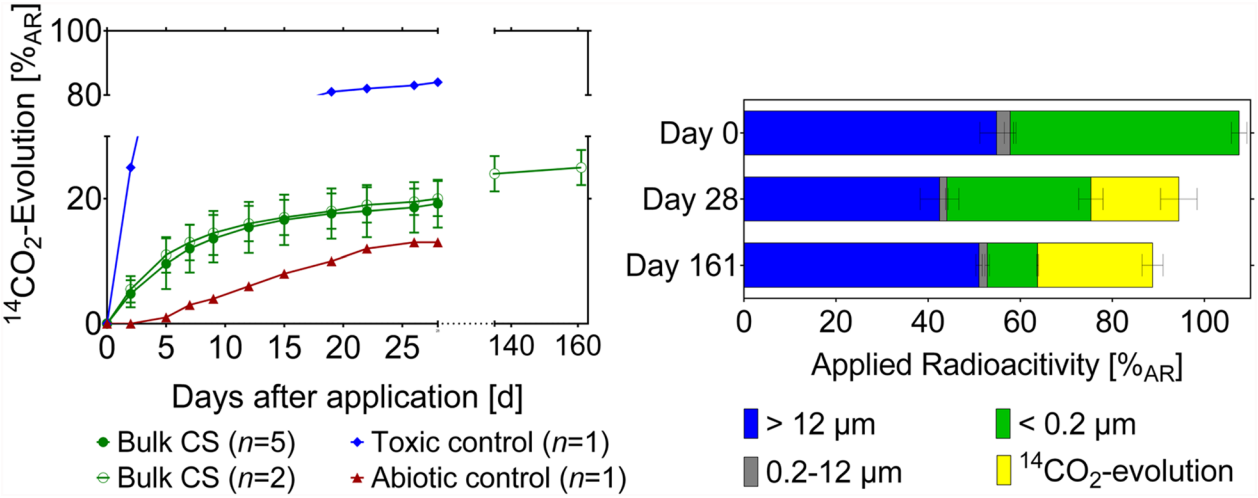


Fig. 2 Biodegradation test results of the fist OECD TG 301B. Left: $^{14}CO_2$-evolution of the bulk polyurea capsule suspension (Bulk CS), the abiotic control (sodium azide-treated) and the toxicity control (sodium benzoate added to the bulk CS). Plotted is the cumulative portion of applied radioactivity (AR) mineralized to $^{14}CO_2$ relative to the incubation time (mean and SD). Right: applied radioactivity (AR) on the different pore-sized filters and in the filtrate at day 0, at day 28 ($n = 5$) and at day 161 ($n = 2$) of test samples after the sequential filtration (mean and SD). Residues remaining on the 12-µm pore-sized filter are displayed as " > 12 µm" and "0.2 to 12 µm" represent the sum of the AR found on the three pore sized filters (0.2, 0.45 and 5 µm). The radioactivity in the filtrate is displayed as the fraction " < 0.2 µm"

the mineralization, which was also observed in a study of Süßmuth et al. [18] for soil samples. Investigations of Blaschko et al. [17] showed that the inhibition by sodium azide can be reversible. Furthermore, a study of Hendrix et al. [19] showed that the addition of sodium azide to environmental samples can lead to reactions with dissolved organic matter causing a lowered concentration of the inhibitor in the solution.

The biodegradation test of the bulk CS resulted in 19.1 ± 3.96%$_{AR}$ (average and stdev of $n = 5$) $^{14}CO_2$-evolution after the standard incubation time of 28 d (Fig. 2). A negligible amount of $^{14}CO_2$ (carbonic acid ($H_2{}^{14}CO_3$)) was dissolved in the sample, as from the two bulk CS samples no $^{14}CO_2$ evolved after acidification with HCl (23.0 ± 1.22%$_{AR}$, $n = 2$). The two bulk CS samples that were incubated for 161 d reached 25.0 ± 2.29%$_{AR}$ (mean of $n = 2$, range). Considering that PUA is a rigid and stable material (see also section "Radioactive labeling and capsule suspension synthesis"), this relatively high degree of mineralization (approx. 40% of the pass criterion for biodegradability using this test method (OECD TG 301B, group 1 and 2)), was unexpected. Initially, it was hypothesized that the observed mineralization may have resulted from the structural modification of the polymer, specifically the inclusion of additional ester bonds intended to enhance biodegradability. Since a crucial biodegradation mechanism is the hydrolysis of ester bonds to liberate monomers [20, 21], the moderate (bio) degradation may have been a result of this modification. However, as demonstrated in subchapter "Biodegradation of the different size fractions of the CS", this assumption was incorrect, and the main component that was mineralized—aminocaproic acid—was identified.

Biodegradation of the different size fractions of the CS

The sequential filtration experiments provided further insights into the (bio)degradation of the bulk CS (Fig. 2) by approximating the size distribution of the bulk CS, not only at the start of the experiment, but also during the incubation time within the OECD 301 biodegradation test.

Generally, the radioactivity was found to fall almost completely in two fractions of the applied sequential filtration approach for the bulk CS at the test start (0 d): 54.9 ± 3.64%$_{AR}$ was found on the 12-µm filter and 49.7 ± 1.67%$_{AR}$ was found in the filtrate < 0.2 µm (0 d, Fig. 2), while the summed up AR of the size range in-between 0.2 and 12 µm, remaining on the 0.2, 0.45 and 5 µm filters, was low (2.93 ± 1.26%$_{AR}$). Thus, slightly more than half was larger than 12 µm as expected, in line with the results of a laser diffraction analysis (SI—Table S3) revealing a median size of the bulk suspension of 16.5 µm (measured size range 0.01–5.75 µm; SI—Table S2, Table S4 and Fig. S2), whereas almost half was present in the filtrate.

As mentioned above, 19.1%$_{AR}$ evolved to $^{14}CO_2$ at 28 d for the bulk suspension (Fig. 2). The radioactivity that had been released from the test medium by mineralization (0 d to 28 d) was presumably reflected in the reduction of radioactivity in the smallest size fraction with a decrease of 18.4%$_{AR}$ resulting in a total remaining activity of 31.3

± 2.64%$_{AR}$ recovered 28 d after application (Fig. 2). In this context, the radioactivity in the fraction between 0.2 to 12 µm was only reduced by approximately 1%$_{AR}$ down to a total of 1.58 ± 0.28%$_{AR}$ (Fig. 2). Notably, the largest fraction (> 12 µm) of the sample—attributed to ^{14}C-labeled microcapsules—also decreased in the amount of radioactivity from d 0 to d 28 by 12.4%$_{AR}$ (total of 42.5 ± 4.22%$_{AR}$ at d 28, Fig. 2). This decrease raised the question whether also the largest particles (presumably microcapsules) were partly degraded to smaller fragments, molecules or even mineralized.

Finally, the sequential filtration results of 161 d incubation showed that the smallest size fraction (filtrate < 0.2 µm) decreased by a total of 38.8%$_{AR}$ compared to d 0, ending at 10.9 ± 0.18%$_{AR}$ (Fig. 2). However, this reduction in radioactivity exceeded the amount mineralized to $^{14}CO_2$ (25.0 ± 2.29%$_{AR}$ from d 0 to d 161). This missing radioactivity in the smallest fraction may be attributed either to a loss of $^{14}CO_2$ through leakage or to the adsorption of radioactivity onto larger agglomerates. Given the lengthy biodegradation period and the complex sequential filtration process, an overall recovery of 88.7%$_{AR}$ at 161 d remains high. The minor observed losses might be attributed to the smallest fraction. Yet, the missing AR in the smallest size fraction might also result from sorption or assimilation into the largest size fraction, retained by the 12-µm filter, which increased by 8.57%$_{AR}$ from d 28 to d 161, reaching a total of 51.1 ± 0.69%$_{AR}$ (n = 2, Fig. 2). Microorganisms of the test medium may have assimilated a portion of ^{14}C-compounds from the smallest size fraction. The living and dead microorganisms might stick together as a microbial aggregate or biofilm which might have reached sizes above 12 µm. Thus, theoretically these microbial aggregates containing assimilated ^{14}C were retained by the 12 µm filter along with the microcapsules to cause an 8%$_{AR}$ -increase in the size fraction > 12 µm after an incubation of 161 d. Supporting this, an increasing amount of flocculant was observed in the sample vessels (SI—Fig. S6). Further investigations, e.g., by analyzing the filter residue or the biofilm aggregates for ^{14}C-lipids, ^{14}C-proteins or ^{14}C-nucleic acid, which can be clearly differentiated from ^{14}C-microcapsules > 12 µm, could be useful to be implemented in future biodegradation testing. For instance, approaches similar as reported by Weng et al. [22] and Zumstein et al. [23] might be useful for characterization of the ^{14}C-uptake of microbial cells, which is also proposed by Zumstein et al. [24] to be considered for biodegradation assessment of plastics. In addition, the proportion of AR in the size fraction between 0.2 and 12 µm remained stable compared to 28 d with 1.77 ± 0.49%$_{AR}$ at 161 d (Fig. 2), indicating that no relevant fragments emerged in that size range.

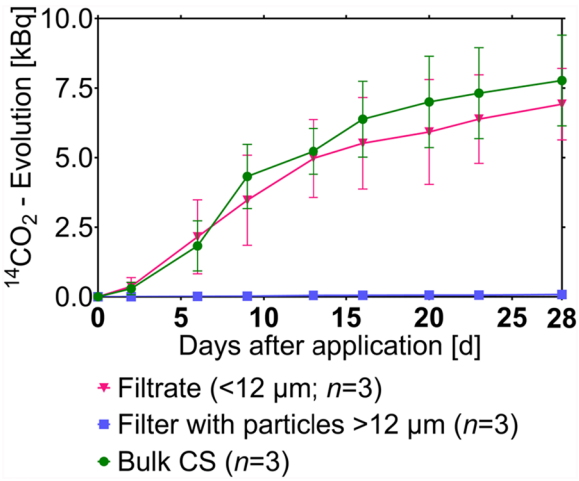


Fig. 3 Biodegradation test results of the second OECD TG 301B. $^{14}CO_2$-evolution of the Bulk CS and its separated size fractions: filter with particles > 12 µm and filtrate with compounds < 12 µm in the second OECD TG 301B. Plotted is the cumulative radioactivity (kBq) that was mineralized to $^{14}CO_2$ relative to the incubation time (mean and SD of n = 3)

The CS was submitted to a second OECD 301B biodegradation test (Fig. 3). Since the radioactivity found in the fraction > 12 µm decreased from d 0 to d 28 within the sequential filtration, the aim was to investigate whether particles (> 12 µm) were mineralized or whether the only source of mineralization was from lower size fractions and the decrease found in the largest size fraction originated from fragmentation or the imprecision of the method. Therefore, in this test, the CS was separated into two size fractions prior to biodegradation testing (< 12 µm and > 12 µm). The compounds and particles < 12 µm were in this approach mineralized to a similar degree as the bulk suspension (deviation by a factor of 1.1, $^{14}CO_2$-evolution of 6.92 ± 1.28 kBq for filtrate < 0.2 µm *vs* 7.77 ± 1.63 kBq for the bulk suspension; Fig. 3). However, for the size fraction of > 12 µm no mineralization to $^{14}CO_2$ was observed (0.08 ± 0.02 kBq). Hence, this experiment clearly showed that biodegradation with respect to $^{14}CO_2$ evolution largely originated from particles, mono- and oligomers with sizes below 0.2 µm instead of the PUA microcapsules.

Ultra-high performance liquid chromatography with high resolution mass spectrometry (UHPLC–HR-MS)

It was assumed that a higher fraction of radioactivity of the bulk suspension might be present in ^{14}C-molecules or ^{14}C-oligomers remaining from synthesis rather than ^{14}C-particles that were mineralized. The UHPLC–HR-MS analysis (total range of 100–6000 m/*z*) coupled with a radio detector (SI—Tables S6, S7, S8) was thus

employed to investigate of the filtrate (< 0.2 µm) of the pristine bulk suspension, which was used as the application solution. It was aimed to identify which components of the smallest particle size fraction (< 0.2 µm) of the bulk suspension might have been biodegraded to $^{14}CO_2$.

In this analysis, labeled and non-labeled aminocaproic acid (*m/z* range of 132.10125–132.1057; SI—Fig. S7) was detected based on the determined molecular formula using the Xcalibur Qual Browser (Version 4.0) in the HR-MS analysis. This molecule is the oxidized derivative of the ^{14}C-labeled monomer ^{14}C-hexamethylenediamine used for synthesis, containing a carboxyl group. For verification, HR-MS analysis of pure (≥ 99%) aminocaproic acid as a reference compound (10 ng/mL, Sigma-Aldrich Chemie GmbH, Germany, SI—Fig. S7) was performed. Focusing on the eluted radioactivity, within the radiodetection analysis of the eluate, the main radioactivity peak was detected in the same RT range (SI—Fig. S7) as the eluted aminocaproic acid. Furthermore, by means of the column recovery, the amount of radioactivity that eluted during the same RT as the aminocaproic acid peak was quantified. In this analysis, it could be demonstrated that 83.3 ± 2.29% (average of $n = 3$; SI—Fig. S8) of the injected radioactivity, more precisely of all ^{14}C-labeled molecules or oligomers introduced to this analysis, eluted at the same RT as aminocaproic acid (RT including both peaks from 3.5 to 7 min—Fig. S7).

Using the isotopic pattern analysis, all molecules or oligomers that consist of the same isotopic pattern of the discovered aminocaproic acid were extracted to a chromatogram. In this chromatogram (SI—Fig. S3) no other molecules or oligomers containing aminocaproic acid as building block eluted, since only the two consecutive peaks were present. This gives further evidence that aminocaproic acid constituted a high fraction of all ^{14}C-labeled molecules that were present in the filtrate of the CS (< 0.2 µm). Besides, the emerging two peaks of aminocaproic acid were presumably due to a part of the molecules being present in an ionized form and therefore more polar causing a faster elution. The other part was uncharged and therefore adhered more strongly to the non-polar column, prolonging RT.

Taken together, our analyses strongly implied that aminocaproic acid was present in high fractions above 80% in the suspension's filtrate (< 0.2 µm) attributable to the oxidized form of the hexamethylenediamine used for the synthesis of the PUA shell of the capsules. The proportion of radioactivity quantified within the column recovery (< 0.2 µm; 83.3 ± 2.3% of the injected radioactivity), that was attributable to aminocaproic acid, was then extrapolated to the radioactivity of the bulk suspension by multiplying this proportion with the relative amount of the bulk suspension's radioactivity in the filtrate (49.7 ± 1.67%$_{AR}$), resulting in approx. 41%$_{AR}$ of aminocaproic acid being dissolved in the bulk suspension. Therefore, presumably most of the observed mineralization originated from the degradation of aminocaproic acid. Supporting this deduction, aminocaproic acid is classified as readily biodegradable according to its safety data sheet with 76% mineralization in 28 d (OECD TG 301D [25]). However, the synthesized polymeric shell did not only consist of the labeled HMDA, but also of components of the co-polymer isocyanate and general crosslinked undefined, random structures. By labeling one monomer, the mineralization and tracking was focused on compounds and building blocks consisting of this monomer. The fate of compounds or molecules consisting of building blocks from (non-labeled) other parts of the polymer was not addressed in this study.

Methodological considerations for microplastic biodegradability testing: a comparative perspective

Our findings emphasize the necessity of characterizing and purifying polymer particles before biodegradation testing, as soluble low-molecular-weight residues can bias the derived mineralization rates. This outcome aligns with Karagianni et al. [26], who demonstrated that depending on the extraction of the polymeric shell and their purification method from the oil and soluble and insoluble ingredients of a microcapsule used for fragrances, the biodegradation test results (OECD 301 F [25]) largely differed, underscoring the necessity of eliminating non-polymeric components prior to testing.

Given the complexity of MP biodegradation assessments, radiolabeling provides distinct advantages for method validation, precise quantification and mass-balancing of polymer-derived mineralization. The advantages of isotope labeling in tracking the biodegradation and mineralization of polymers or polymer fractions of a product were highlighted by a study on radiolabeling approaches for polymer degradation [27]. Such labeling is particularly useful for tracking difficult-to-detect polymer particles, such as microcapsules with low polymer content. However, the high synthesis costs and the need for specialized laboratories with respective license and specific analytical instrumentation limits its application to applied research and method validation rather than routine testing. Once alternative endpoints are identified and methods are established, testing should ideally be possible without radiolabeling. Alternative analytical techniques might support here. Fourier transform infrared (FTIR) and Raman spectroscopy could be used to analyze polymer particles at different stages of biodegradation, providing

chemical characterization throughout the process. Scanning electron microscopy (SEM) can offer valuable insights into morphological changes and fragmentation. However, the feasibility of replacing radiolabeling with these methods depends on potential matrix interferences, MP particle size and their detection sensitivities as well as the availability of those analytical methods for routine testing.

Furthermore, recent studies have indicated size-dependent biodegradation of synthetic polymer particles showing that smaller MPs degrade more efficiently due to their higher surface area-to-volume ratio, enhancing microbial colonization and impact of enzymatic activity [28–34]. These findings support the need to integrate size monitoring alongside mineralization endpoints in biodegradability assessments.

Conclusion

This study supports two major recommendations for future biodegradation testing: Firstly, the tested material needs purification to exclude potential bias from the biodegradation of impurities or alternatively, a comprehensive characterization of the bulk product needs to be conducted. Secondly, monitoring the size distribution of the tested microparticles is useful, to obtain a more comprehensive picture including primary biodegradation. Integrating additional parameters such as particle size and the release or presence of bioavailable compounds gives more insights into how MPs degrade. The parameters substantiate the biodegradation test results within regulatory assessments to support accurate and valid classifications regarding MP degradability.

The UHPLC–HR-MS analysis of this study revealed that the apparent CS biodegradation was biased due to radiolabeled, low-molecular weight, soluble residues from polymer synthesis that caused the $^{14}CO_2$ evolution reaching 29%$_{AR}$ (day 161). We demonstrated that microcapsules from an aqueous suspension can be isolated (purified) by filtration with a rinsing step and subsequent resuspension in water. This procedure could also be applicable for non-labeled biodegradation testing.

The results demonstrate that ^{14}C-labeling is a powerful tool for distinguishing the polymer biodegradation from the mineralization of other constituents, in particular if the tested particles are more complex and contain more than the polymeric material. In addition, ^{14}C-labeling facilitates identification and quantification of compounds of interest, if further specific analyses coupled to radiodetection, such as UHPLC–HR-MS analysis, are implemented.

Abbreviations

PUA	Polyurea
CS	Capsule suspension
AR	Applied radioactivity
HCl	Hydrochloric acid
LSC	Liquid scintillation counting
MP	Microplastic
NaOH	Sodium hydroxide
RT	Retention time
TC	Total carbon
TG	Testing guideline
UHPLC–HR-MS	Ultra-high performance liquid chromatography with high-resolution mass spectrometry

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-025-01096-8.

Additional file1

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Author contributions
EMT: conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing the original draft and editing of reviewed manuscript; JH: UHPLC–HR-MS analysis investigation and evaluation; BM: methodology; AJ, AS, BM: writing—review and editing. AJ, AS, BM, DH, MS, PD, HE, RH: supervision. AS, BM, DH, MS, PD, HE: project administration, funding acquisition. All authors revised and edited the manuscript and approved the final version before submission.

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Availability of data and material
The data used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest
Philipp Dalkmann and Holger Egger are employees of the Bayer AG Division Crop Science, a leading manufacturer of agricultural chemicals. Roman Heumann is and Eva-Maria Teggers was employed at INVITE GmbH, Germany, a cross-industry company with 50% business shares belonging to Bayer AG and 50% business shares belonging to universities (Technical University of Dortmund, Germany, and Heinrich Heine University Düsseldorf, Germany).

Ethical approval and consent to participate
Not applicable.

Consent for publication
All authors consent for the publication of this work. They have reviewed the final manuscript for accuracy.

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