

Article

Selective Enrichment of Fibrous Fragments Formed from Milled Carbon Fibers by Means of Gravitational Settling in a Liquid

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Highlights

What are the main findings?

- A procedure combining ball milling, liquid suspension, ultrasonication and gravitational settling is introduced that enables to prepare a material sample of polyacrylonitrile-based carbon fibers in order to maximize its concentration of critical WHO-analog fibers for the performance of toxicological tests.
- The number of critical WHO-analog fibers per total sample mass was enhanced from 0.36×10^6 to 6×10^6 fibers/mg and is therefore suitable for intraperitoneal testing in rodents according to the applicable guideline EUR 18748 EN and the German Technical Rule for Hazardous Substances No. 905.

What is the implication of the main finding?

- The results of subsequent toxicological studies using our material prepared according to the described procedure are expected to have high regulatory relevance, as they provide scientific data required for the development and implementation of a coherent regulatory framework for critical fibers.
- The use of semi-automated software tools and artificial neural networks developed in house facilitates the detailed and statistically representative morphological characterization of particle- and/or fiber-containing material samples in the future and represents a fundamental progress for the assessment of health risks related to quantified biopersistent respirable fiber fractions in dusts emitted.



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Abstract: The aim to reduce health risks of workers related to inhalative exposure to potentially toxic dusts requires the selection of appropriate measures depending on the hazard classification of the dust-composing materials. Due to their biodurability, respirable carbon fibers and their fragments can impose such health risks but are currently lacking hazard classification. Here, a method is presented for fragmenting carbon fiber materials and enriching fibrous fragments to a level that is expected to allow differentiating between fiber and particle overload-related toxic effects. The method was applied to a commercial polyacrylonitrile-based carbon fiber. It was ground in an oscillating ball mill, homogenized in a liquid using ultrasonication and left undisturbed for gravitational settling. This way, a vertical gradient in particle size and shape formed, from which the supernatant was collected. Fragment morphologies were characterized with large ensemble statistics by semi-automated evaluation of scanning electron microscopy images employing an artificial neural network for binary semantic segmentation. The number of fibrous fragments of respirable and thus critical fiber morphology was increased from 0.36×10^6 to 6×10^6 WHO-analog fibers per mg. This corresponds to a factor of about 15 compared to the initial ground material. Since the mass percentage of non-fibrous objects was also significantly reduced,

the requirements for a subsequently scheduled toxicological study with intraperitoneal application were fulfilled. Intraperitoneal testing is an accepted method for assessing the carcinogenic potential of biopersistent fibers. The developed method allows enriching fibrous fractions of concern at acceptable throughput and enables testing fiber toxicological effects of respirable fragments from disintegrated polyacrylonitrile-based carbon fibers.

Keywords: PAN carbon fiber; sedimentation; toxicological testing; occupational health; artificial neural network; WHO fibers

1. Introduction

Carbon fibers (CF) are mainly used in composite materials for lightweight high-performance structural components [1]. Compared to other precursors, polyacrylonitrile-based (PAN) CF are the most widely used variant and are produced in high volumes [2]. They are spun from polymer solution with resulting diameters of typically greater than 4 μm and are thus initially non-respirable by design. However, despite their excellent mechanical properties, structural failure of CF or CF-containing composites can occur during manufacture, handling, use and recycling. Carbon fiber disintegration results in the formation of granular and fibrous fragments that are of concern to human health if respired at critical doses.

In contrast to the widespread use of CF, there are only limited data from epidemiological and toxicological studies on the hazard of CF fragments and only sparse data on exposure to CF-derived dust [3–12]. Consequently, no CF-specific occupational exposure limits (OELs) have been implemented. However, being carbonized and partially or highly graphitic materials, CF are inorganic materials that must be considered to exhibit high biodegradability. Accordingly, respirable fibrous and granular particles contained in CF-derived dusts can persist and accumulate in lung tissue. Granular CF dusts are therefore subject to general occupational hygiene regulations for inert dusts. Such regulations aim at preventing overexposure-related health effects by imposing mass-based exposure limits. Fibrous CF fragments are to be treated as inorganic fiber dusts with, in many countries, mandatory fiber number-based exposure limits. For example, the German “Technical Rules for Hazardous Substances” (TRGS 905) [13] recommend that all inorganic fiber dusts containing respirable fiber-like particles with specific dimensions of concern in terms of width, length and aspect ratio are to be classified as suspected carcinogenic according to category 2 of the European CLP Regulation (“Classification, Labelling and Packaging”) [14], unless sufficient toxicological data indicate non-toxicity. A material classified in category 2 can be declassified with a negative test result at both a dose of 1×10^9 and a higher dose of 5×10^9 fibers per rat using intraperitoneal testing [13]. To classify a material as presumably carcinogenic according to Category 1B (“Presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.”), the corresponding guideline for toxicological testing [13] requires a positive test result at a dose of 1×10^9 fibers.

The specific fiber dimensions of concern include fibers with a length $L \geq 5 \mu\text{m}$, a width $W \leq 3 \mu\text{m}$ and a length-to-width aspect ratio greater than 3. These limits are also referred to as “WHO dimensions”, as they include those fibers that are to be counted according to the WHO fiber counting convention [15]. However, since the WHO counting convention must also be executable with phase-contrast optical microscopy, it does not count fibers thinner than 0.2 μm . The term “WHO-analog” fibers will therefore be used subsequently.

In general, the tendency of a material to form fiber-like fragments depends on both the intrinsic anisotropic structural properties of the material and directional aspects of the disintegration-causing processes. Due to the increasing production, compounding and

use of CF, a risk-based material classification seems necessary. In particular, the ongoing upscaling of the recycling of CF-reinforced composites, including technologies such as chemolysis, pyrolysis, shock wave application or Joule heating [16], leads to new release scenarios and potentially higher occupational exposure to CF-derived dusts.

In order to comply with occupational hygiene requirements, both an improved understanding of the toxicology of CF-derived dust particles and the efficacy of measures to control exposure to biodurable respirable fiber dust are necessary. While occupational risk assessment considers both the respired dose resulting from the release and exposure to fiber dusts, as well as fiber dust-specific hazards, hazard assessment alone is not concerned with the likelihood of fiber fragments formation but whether it can occur at all.

In intraperitoneal (i.p.) testing, a fiber suspension is injected into the peritoneum of rodents. The animals are subsequently observed for two years and investigated histopathologically for the occurrence of mesotheliomas. According to Ref. [17], all asbestos fiber types that caused mesotheliomas in humans, as well as all asbestos and manmade mineral fiber types that caused mesotheliomas in experimental animals after inhalation exposure, also caused mesotheliomas after i.p. injection in rats [18–24]. Despite the long history of fiber toxicological testing, the selection of appropriate tests is subject of ongoing discussion [25,26]. Some authors have reported a lower sensitivity of rats towards asbestos in inhalative compared to intraperitoneal application (see Figure 3 in Ref. [27]).

Although the systemic administration of fibers and particles bypasses physiological exposure pathways, it is widely accepted that direct intravenous or intraperitoneal injection of particles serves as a reproducible, valid and convenient method for investigating translocation mechanisms and biological responses [28].

Intraperitoneal tests in rats are considered specific for the carcinogenicity of biopersistent fibers and are accepted by EU regulatory authorities to investigate whether a fiber material can induce pleural or peritoneal tumors [29]. In contrast to fibers, biopersistent granular dusts were not able to induce mesotheliomas in this test, even when applied at very high doses of 4×20 mg per rat [30].

Test materials to be used for in vivo or in vitro toxicological studies that aim at identifying the hazards of a specific material must meet very high standards with respect to chemical and morphological characterization, endotoxin contamination, etc., especially when selected for inhalative, instillative or intraperitoneal application in rodents, also for ethical reasons. Even more challenging are the requirements for materials that allow differentiation between fiber and particle overload-related toxic effects in in vivo application, as the dose ratio of fibers to granular particles must be carefully controlled. This requires the laborious compilation of statistically representative morphological distributions of fragment length and width by means of microscopic analysis. In addition, the guideline for i.p. testing limits the total injected mass to a maximum of 250 mg per animal [29]. A test material must therefore provide a dose of at least 4×10^6 fibers of WHO-analog dimensions per mg sample material for the lower test dose of 1×10^9 fibers per animal. Since i.p. studies are usually performed with at least 50 animals per dose level, this can result in a demand for the test material on a multigram scale. This excludes small-scale separation techniques and requires a moderate-to-high separation yield, along with a reasonable throughput per separation run.

Various techniques have been developed to generate fibrous test material from particulate matter (PM) for use in toxicological testing, including the use of sophisticated electrostatic precipitators as PM collectors [31,32] and of differences in gravitational settling, as were reported for airborne microplastic fibers [33]. Ball milling was found to be a less sophisticated but still effective source of fibrous CF debris when applied to various CF materials [34]. Both PAN-based and mesophase pitch-based CF disintegrated into fibrous

fragments. Ball milling of a highly graphitic pitch-based CF produced a material with such a high content of WHO-analog fibrous fragments that it was directly subjectable to a fiber toxicological i.p. study in rodents. A publication on the results of this study is currently in preparation [17]. PAN-based CF showed a smaller but still significant tendency to form WHO-analog fragments compared to pitch-based CF [34].

The present work reports the results of a systematic study to enrich the fraction of WHO-analog fibrous fragments in ball-milled PAN-based CF samples to a level that allows to supply test material for toxicological i.p. studies that aim at the differentiation of fiber- and particle-related toxic effects. The range of process parameters that could be investigated in the present work for process optimization was comparatively large, as the laborious compilation of morphological distributions was supported by automated microscopic image acquisition and semi-automated fragment analysis.

2. Materials and Methods

2.1. Materials

Commercially available rovings of a PAN-based CF of type Tenax-E HTS40 F13 K12 with a density of $\rho_{CF} = 1.77 \text{ g/cm}^3$ and a monofilament diameter of $7 \mu\text{m}$ were provided free of charge by Teijin Carbon Europe GmbH (Heinsberg, Germany). This type of PAN-based CF was chosen because of its high applicational relevance and its tendency to produce more fragments of concern than other previously tested PAN-based CFs [34].

2.2. Methods

Manually pre-cut carbon fiber chops were disintegrated using an oscillating ball mill. The ground samples were suspended in a liquid and homogenized using ultrasonification with a sonotrode. The mixture was then left undisturbed to settle by gravity. After a definite time, the supernatant of the suspension was collected. The contained solid was collected by filtration using polycarbonate membrane filters clamped to a vacuum filter holder. Samples for scanning electron microscopy (SEM) analysis were filtrated on gold-coated membrane filters. Details are provided in the following sections.

2.2.1. Structural Disintegration by Milling

An oscillating ball mill of type MM200 (Retsch GmbH, Haan, Germany) with 10 mL zirconia grinding jars and two zirconia grinding balls of 10 mm in diameter (Retsch GmbH, Haan, Germany) per jar were used to grind the PAN-based CF material for 22 min at a vibration frequency of 9 Hz. Each jar was filled with a total of 24 cm of the PAN-based CF roving cut into approximately 1 cm pieces. Between two grinding runs, the jars and balls were cleaned with dry, low-linting tissue paper.

As reported earlier, the milling of carbon fiber roving cuts with a total length of only 8 cm for about 90 s and with a frequency of 25 Hz provided a very finely ground material and a first estimation of the maximum proportion of WHO-analog fibers (WHOFO) of 2% with respect to the total number of fragments [34]. As a first measure, the frequency was therefore reduced to 9 Hz. This made it necessary to also prolong the milling duration to 8 min to obtain a pourable ground material. By this, the proportion of WHOFO could be increased to about 13%. In order to minimize the number of required milling processes to provide the required amount of test material, the CF quantity milled was tripled. Since this again reduced the milling energy transfer per CF mass, the length and diameter distribution of the resulting ground material changed, and the milling duration was adjusted accordingly. A milling duration of 15 min resulted in only 3% WHOFO per all fragments. By further increasing the milling duration a maximum of up to 17%, WHO-analog fibers could be identified after 22 min of milling. Another important criterion for evaluating

the grinding process was a good pourability of the resulting dry material, which was required for its handling, weighing and dispersing in liquids. Short milling times yielded a strongly tangled product that could not easily be handled. Longer milling times yielded a fine-grained product and gradually decreased the resulting fragment size. Milling of 24 cm for 22 min produced the material with the highest observed proportion of WHOFO with respect to the total number of fragments. It was used for the subsequent investigations.

Alternatives to the milling process were likewise investigated, including a modified apparatus for sandblasting and, for wind sifting, a vibro-fluidization device [35]. The former suffered from orifice blocking and low disintegration efficiency, and the latter yielded no significant WHO-analog fiber enrichment, probably due to insufficient fluidization in the rather short wind sifting column. In addition, as aerosol processing is more critical from an occupational safety point of view than for gravitational settling in liquids, it was depreciated.

2.2.2. Suspension and Sampling of Milled PAN-Based CFs for Morphological Analysis

A suspension of at least 5 mg of the ground PAN-based CF was prepared with 10 mL 2-propanol (Rotipuran grade, Carl Roth, Karlsruhe, Germany). To the experience of the authors, 2-propanol of Rotipuran grade is well suited as a suspension medium for CF fragments. In addition, 2-propanol of the selected grade evaporated without residues, which is important for the subsequent i.p. testing.

An aliquot of 0.1 mL of the prepared suspension, containing a minimum of approximately $m_F = 50$ g ground CFs, was taken with a pipette from the center of the freshly shaken vial under magnetic rod stirring before any visible sedimentation of larger fragments occurred. That aliquot was then added dropwise into the funnel of a clamped vacuum filter holder (Merck Millipore, Darmstadt, Germany), which had previously been filled with about 15 mL of 2-propanol. After rinsing the pipette tip, the resulting suspension in the funnel was stirred and vacuum-filtrated on gold-coated track-etched membrane filters of 25 mm in diameter and a 400 nm pore size (APC GmbH, Eschborn, Germany) using two support filters (deconta GmbH, Isselburg, Germany). No metal frit was used as filter support, as such frits were observed to lead to an inhomogeneous deposition. Up to 15 mL of 2-propanol was used for rinsing the funnel. Loaded filters were subsequently imaged by SEM as outlined in Section 2.2.5.

2.2.3. Homogenization, Subsequent Gravitational Settling and Collection of Supernatants

A volume of 800 mL of 2-propanol of Rotipuran grade was placed in a glass beaker of 1000 mL in volume and an inner diameter of 10 cm. An ultrasonic tip (Microtip CL134) was positioned approximately at the 200 mL mark. After dispersing a mass of 1.6 g of milled PAN-based CFs with a magnetic rod coated with PTFE in the 2-propanol, the ultrasonic probe (model FB705, Fisher Scientific, Pittsburgh, PA, USA) was operated for 15 min at an amplitude of “100”, resulting in a total output of about 52,000 J. Detailed information on how the ultrasonication duration affected the particle size distributions and the yield of WHO-analog fragments is described in Section 3 and discussed in Section 4.

The resulting suspension was left standing for 35 min. Without further delay, the supernatant liquid of 700 mL in volume was gently collected by suction through a chemically resistant tube. The remaining lower volume was discarded. The collected supernatant was dispersed by manual vigorous shaking prior to taking an aliquot of 0.1 mL in volume for an optional SEM analysis of the batch. The remaining suspension was vacuum-filtrated using a clamped vacuum filter holder (Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany) on a 50 mm polycarbonate membrane filter (400 nm pore size, Whatman Nucleopore Cytiva). This yielded a filter cake, which was vacuum-dried at a reduced pressure of

50 mbar at room temperature overnight. On average, about 115 mg sedimentation-refined material was obtained from the supernatant.

In total, 147 repetitions of this procedure were necessary to provide the requested amount plus reserve material for the planned subsequent toxicological study with i.p. application. To reduce the consumption of 2-propanol, the solvent obtained from filtering the supernatant was additionally filtrated through a track-etched polycarbonate membrane filter of 50 mm in diameter and 200 nm in pore size (Carl Roth, Karlsruhe, Germany) and reused in the subsequent run. Samples from reused 2-propanol were rinsed with fresh 2-propanol after filtration and before drying. Prior to shipping the test material to partners performing i.p. tests, all filtered and rinsed materials were dried at room temperature to remove any remaining 2-propanol.

During the settling of a particle suspension in the 2-propanol fluid of a density ϱ_{2p} and a dynamic viscosity η_{2p} , a separation of different particle sizes occurs over time. Each particle settles with a velocity depending on its density $\varrho_{CF} = 1.77 \text{ g/cm}^3$, shape and orientation, which determine its equivalent-dynamic diameter.

For an elongated particle of length L and width W with $L > W$, which settles being vertically oriented, i.e., parallel ($\parallel$), perpendicular ($\perp$) or random to the settling direction, the corresponding equivalent sphere diameters D_{eq} are [36,37]

$$D_{eq\parallel} = W \cdot \sqrt{\frac{9}{4} \ln\left(2 \frac{L}{W}\right) + \ln(2) - \frac{3}{2}} \quad (1)$$

$$D_{eq\perp} = W \cdot \sqrt{\frac{9}{8} \ln\left(2 \frac{L}{W}\right) + \ln(2) - \frac{1}{2}} \quad (2)$$

$$D_{eq,random} = \frac{1}{3} \cdot (D_{eq\parallel} + 2 D_{eq\perp}) \quad (3)$$

The orientation of larger cylindrical particles of very regular geometry has been observed to occur perpendicular to the settling direction [38]. Due to the irregular, often wedge-like shape of our fragments, however, an orientation parallel to the settling direction is assumed. According to Stokes' law, for a laminar flow regime and a specific height h , the resulting gravitational settling of a spherical particle of diameter D_{eq} in a fluid would require a time of about

$$t = \frac{18}{g} \cdot \frac{\eta_{2p}}{\varrho_{CF} - \varrho_{2p}} \cdot \frac{h}{D_{eq\parallel}^2} \quad (4)$$

with g being the acceleration of gravity of about 9.807 m/s^2 . Consequently, smaller particles sediment slower than larger ones, and, unless all particles have settled, a particle size gradient results.

After gravitational settling of the suspension for $t = 11 \text{ min}$, the supernatant of a height of $h = 9 \text{ cm}$ was skimmed off, filtered and the filtrate was collected as described above. For this sedimentation duration and height, assuming $\eta_{2p} = 2.3703 \text{ mPa s}$ and $\varrho_{2p} = 786 \text{ kg/m}^3$ for 2-propanol, spherical fragments inside the suspension with a diameter larger than about $D_{esc\parallel}^{11'} = 24.4 \text{ }\mu\text{m}$ can escape the supernatant and reach the residue. When extending the gravitational settling to $t = 35 \text{ min}$, an even higher concentration of WHO-analog fibers was collected (see Table 1). Settling for $t = 35 \text{ min}$ and collecting 700 mL corresponded to a supernatant height of $h = 9 \text{ cm}$ and resulted in a lower escape diameter of spherical particles of $D_{esc\parallel}^{35'} = 13.7 \text{ }\mu\text{m}$.

Table 1. Results of WHO-analog fiber enrichment experiments performing milling and suspending (i), followed by 15 min of ultrasonication (ii) plus 11 min (iii) or 35 min (iv) of gravitational settling for 3 replicate milling batches.

Group	Milling Batch	Ultrasonic Treatment Duration [min]	Settling Duration [min]	Number of WHOFO per mg [10 ⁶]	Group Average WHOFO per mg [10 ⁶]	Group Average $D_{\text{LARPO}}^{95\text{th}}$ ¹ [μm]	Group Average $\langle D_{\text{LARPO}}^{\text{geom.}} \rangle$ ² [μm]	Group Average $\langle W_{\text{HARFO}}^{\text{geom.}} \rangle$ ³ [μm]
i	M1	0	0	0.55 ± 0.05	0.36 ± 0.03	7.2 ± 0.6	2.08 ± 0.34	0.88 ± 0.20
	M2	0	0	0.23 ± 0.04				
	M2	0	0	0.90 ± 0.11				
ii	M2	15	0	0.50 ± 0.07	0.20 ± 0.02	5.7 ± 0.2	1.60 ± 0.07	0.80 ± 0.07
	M2	15	0	0.54 ± 0.07				
	M2	15	0	0.14 ± 0.02				
	M2	15	0	0.60 ± 0.09				
iii	M1	15	11	2.4 ± 0.3	2.65 ± 0.16	5.0 ± 0.2	1.49 ± 0.10	0.74 ± 0.04
	M2	15	11	2.4 ± 0.4				
	M2	15	11	3.8 ± 0.4				
	M3	15	11	2.2 ± 0.3				
	M3	15	11	3.3 ± 0.4				
iv	M3	15	35	6.8 ± 0.9	6.0 ± 0.4	4.9 ± 0.3	1.23 ± 0.09	0.65 ± 0.04
	M3	15	35	5.2 ± 0.7				
	M3	15	35	6.4 ± 0.8				

¹ $D_{\text{LARPO}}^{95\text{th}}$: 95th percentile of LARPO equivalent disc diameters; ² $\langle D_{\text{LARPO}}^{\text{geom.}} \rangle$: calculated geometric mean of LARPO equivalent disc diameters; ³ $\langle W_{\text{HARFO}}^{\text{geom.}} \rangle$: geometric mean from log-normal fitted HARFO widths.

2.2.4. Preparation of Samples Obtained After Ultrasonication and Gravitational Settling for Morphological Analysis

To the 0.1 mL aliquot taken from the shaken supernatant, as described in Section 2.2.3, a volume of 10 mL 2-propanol of Rotipuran grade was added and the resulting mixture given into the funnel of a clamped vacuum filter holder (Merck Millipore, Darmstadt, Germany). After rinsing the pipette tip, the resulting diluted suspension was stirred and vacuum-filtrated on clamped gold-coated track-etched membrane filters of 25 mm in diameter with 400 nm in pore size (APC GmbH, Eschborn, Germany) supported through a support filter (deconta GmbH, Isselburg, Germany). The filter area exposed by clamping had a diameter of 16 mm. After rinsing with 2-propanol and drying, this loaded filter was subsequently imaged by SEM as outlined in Section 2.2.5.

2.2.5. SEM Analysis and Morphological Characterization

Scanning electron microscopic (SEM) analysis of fragments on membrane filters was performed using a Hitachi SU8230 microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany). A total of 50 randomly distributed filter locations were imaged with a size of 5120 × 3840 pixels and a magnification of 800×, corresponding to a pixel resolution of $\Delta P_{800\times} = 31$ nm, at an electron beam energy of 2 keV and a working distance of about 9 mm using in-house developed software for automated stage control and image acquisition. The evaluation of SEM images was done visually by trained personnel, supported by an in-house developed, semi-automated object morphology analysis software (version 1.4.42) [39].

On each acquired image, all visible fibers and particles were marked and counted according to a set of specific counting rules, as described in detail in the Supplementary Information. Briefly, all objects were counted, and the two lateral extensions of their two-dimensional SEM projection image were measured algorithmically, see below, to determine

length L and width W , with $L \geq W$. These morphological data on length and mean width were used to categorize each object either as a high aspect ratio fiber object (HARFO) with $L/W \geq 3$ or as a low aspect ratio particle object (LARPO) with $L/W < 3$. For LARPO, the equivalent disc diameter $D_{eq} = 2/\sqrt{\pi} \sqrt{L \cdot W}$ was calculated. The HARFO class was distinguished further into three disjunct morphological classes: (1) HARFO fragments exhibiting WHO-analog dimensions (WHOFO), (2) HARFO fragments thicker than $3 \mu\text{m}$ (thick non-WHO-HARFO) and (3) HARFO fragments shorter than $5 \mu\text{m}$ (short non-WHO-HARFO).

Figure 1 shows a sample SEM image of CF fragments collected after milling, ultrasonication and gravitational settling. Such images of $\Delta P_{800\times} = 31 \text{ nm}$ pixel resolution and 5120×3840 pixels in size were used for fragment morphology measurement, classification and counting. Typically, the HARFO fragments were of an irregular, often wedge-like shape.

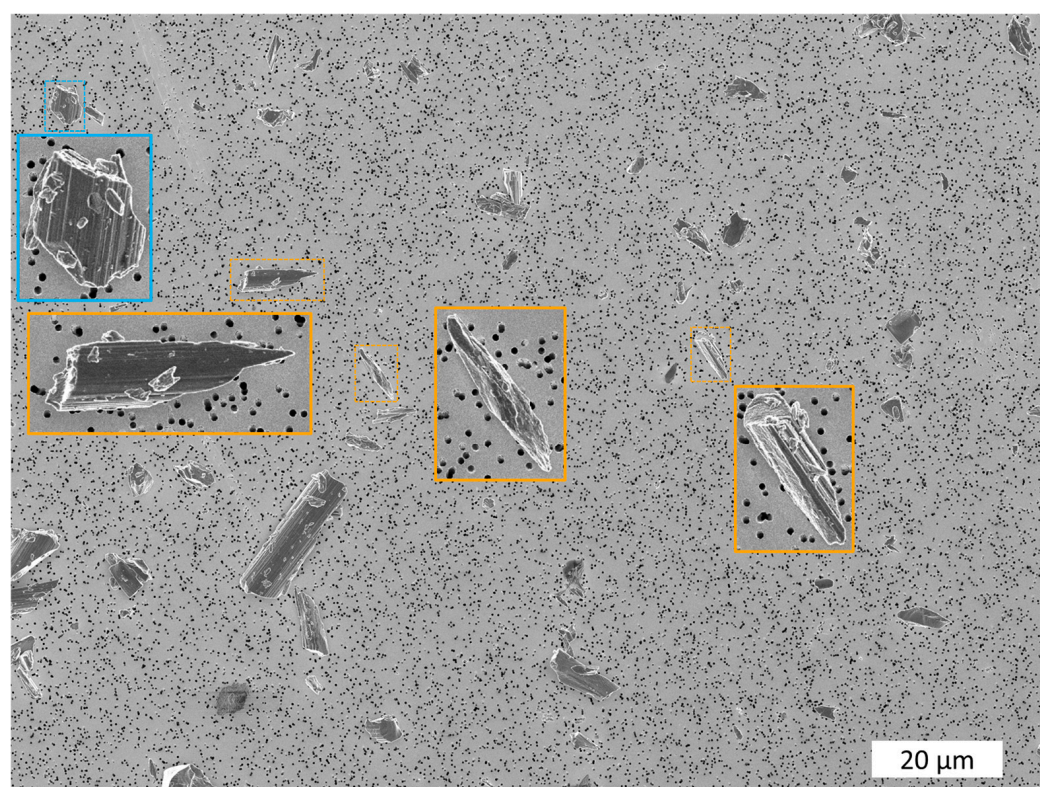


Figure 1. SEM image of carbon fiber fragments after milling, ultrasonication and gravitational settling. The blue and orange dashed frames mark examples of one low aspect ratio particle object (LARPO) and three high aspect ratio fiber objects (HARFO), respectively, which are also shown as solid-line framed insets with a relative magnification factor of 3.

Software-assisted localization and binary semantic segmentation of CF fragments in SEM images were either performed manually, by marking all pixels showing CF fragments using the morphology analysis software, or semi-automatically, by using an artificial neural network (ANN) plug-in to this software (see Section 2.2.6).

The dimensions of all marked fragments were computed algorithmically: the length L being the length of longest straight fiber backbone fully inside a fragment's image, and the mean width $\langle W \rangle$ being the average of measurements of the fragment image's extensions orthogonal to this backbone. Usually, a minimum of 500 objects is evaluated on as many SEM images as necessary to reach this number. Each image was evaluated in its entirety.

For the analysis of CF fragments, an average suspended mass of $m_F = 50 \mu\text{g}$ to $200 \mu\text{g}$ of ground CF was vacuum-filtered on a membrane filter of 25 mm in diameter, of which

only an area with a diameter of approximately $d_F = 16 \text{ mm}$ was left open by clamping. This resulted in a mass area density of about $\delta = \frac{4m_F}{\pi d_F^2} = 0.25 \frac{\mu\text{g}}{\text{mm}^2}$ to $1.00 \frac{\mu\text{g}}{\text{mm}^2}$. At the selected pixel resolution of $\Delta P_{800\times} = 31 \text{ nm}$, one evaluated SEM image of 5120×3840 pixels corresponded to a filter area of $A_E = 0.0189 \text{ mm}^2$ and represented a mass per image of approximately $m_E = A_E \cdot \delta = 4.7 \text{ ng}$ to 18.8 ng . Depending on the actual loading of the filter, in general, a filter area of 0.09 mm^2 to 0.2 mm^2 was evaluated, corresponding to about 5–15 images.

2.2.6. Semi-Automated Evaluation of SEM Images Using Artificial Neural Networks

In order to reduce the workload, SEM images were pre-evaluated using binary semantic segmentation masks predicted by a U-Net model to identify objects. This convolutional autoencoder model was trained on a large dataset of about 1000 manually annotated SEM images of 5120×3840 in pixel size that showed carbon fibers, as well as carbon nanotubes, on track-etched membrane filters. The dataset was divided into two subsets, one for training and one validation using a ratio of 7:1. For data augmentation purposes, we randomly adjusted the image properties of brightness and contrast and rotated and flipped them. The training was stopped when no further improvement in the segmentation quality was achieved for the validation set within a period of 40 epochs. The model's training process is described in detail in Ref. [39].

Although the predicted segmentation was generally very accurate, this automatic segmentation step was always followed by human visual inspection, and manual corrections of individual fragment masks were applied when necessary. Especially, fragments deposited on top of or in contact with one another were separated manually according to the rules described in the Supplementary Information.

Using this advanced image processing tool, in the scope of our process optimization, 93 samples were analyzed, and the length and width of 81,700 segments were determined.

2.2.7. Criteria to Assess the Material Refinement Progress

Since the toxicological regulations of i.p. testing constrain the total injected mass to a maximum of 250 mg per animal, a dose of at least 4×10^6 fibers of WHO-analog dimensions per mg sample material must be provided to reach the lower test dose of 1×10^9 fibers per animal, as described in the Introduction. The number of WHOFO per mass is therefore the central criterion to decide whether a fiber sample is suited for i.p. testing. In addition, a sample should not contain particles that are too large to be respirable. For this reason, there is a recommendation for the 95th percentile of particle diameters of $D_{\text{LARPO}}^{95\text{th}} < 4 \mu\text{m}$. Here, the equivalent disc diameter derived from the two-dimensional SEM image was used as a proxy for the particle diameter.

The following measurands were included in the assessment of morphological properties of the milled material and refined material samples:

- percentage of WHO-analog fibers to the total amount of fragments
- distribution of lengths and widths of LARPO, HARFO and WHOFO, respectively
- geometric mean of lengths $\langle L_{\text{HARFO}}^{\text{geom.}} \rangle$ and widths $\langle W_{\text{HARFO}}^{\text{geom.}} \rangle$ of HARFO
- geometric mean of lengths of WHO-analog fibers $\langle L_{\text{WHOFO}}^{\text{geom.}} \rangle$
- geometric mean of equivalent disc diameters of LARPO $\langle D_{\text{WHOFO}}^{\text{geom.}} \rangle$
- 95th percentile of equivalent disc diameters of LARPO $D_{\text{LARPO}}^{95\text{th}}$

3. Results

The milling and separation procedures were optimized to maximize the number concentration of WHOFO-analog fibers per sample mass while, at the same time, keeping the amount of work for the preparation of the amount of material needed for the *in vivo* study manageable.

WHOFO enrichment experiments were conducted with differently milled specimens, varied sedimentation durations and varied cut-off volumes, as well as with and without ultrasonic homogenization. The results of sequences of up to five replications of experiments varying the ultrasonication and gravitational settling duration are reported in Table 1 and visualized in Figure 2. Replications were conducted to study the reproducibility of the applied sampling and morphological characterization procedures. Upon milling pre-cut PAN-based CF, dispersing them in 2-propanol by shaking and sampling on filters, as described in Section 2.2.2, it was observed that the ground material tended to form agglomerates and covered the filters for SEM analysis inhomogeneously. Representative sampling on filters for SEM analysis proved difficult. Filter sampling and evaluation resulted in large variations of the number of WHOFO per mg sample (cf. the results of group i in Table 1).

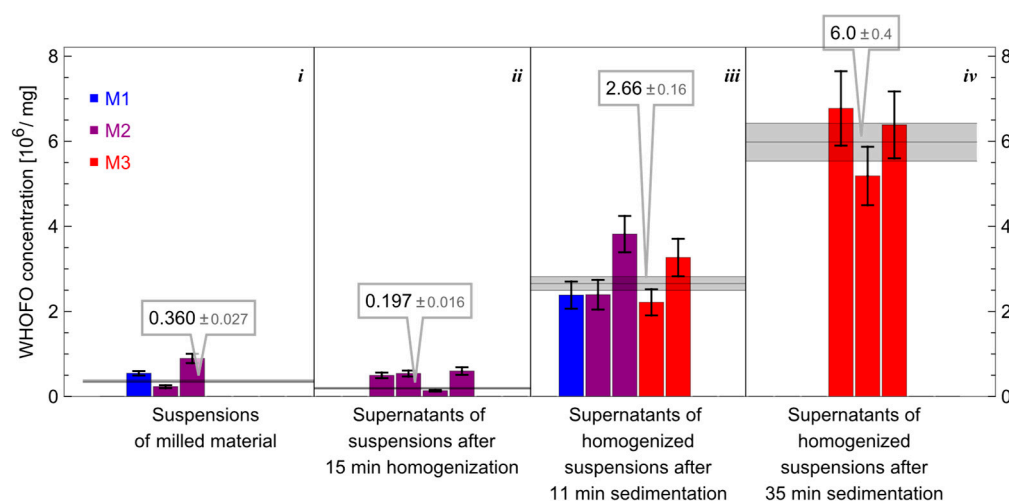


Figure 2. Comparison of up to 5 replications of the process sequence of milling and suspending (i), followed by ultrasonication (ii) and 11 min (iii) or 35 min (iv) of gravitational settling using 3 replicate milling batches: M1, M2 and M3. The callouts and the gray horizontal bar give the error-weighted group averages of the WHOFO concentrations. The legend specifies the initial milling samples used for the replication.

Ultrasonication of the liquid suspension prior to the gravitational settling process improved both the sampling repeatability and the dispersion state. Treatment durations between 0 and 30 min were studied. Long treatment durations of 30 min caused considerable additional fragmentation, as they reduced the average mass of all fragments, especially that of WHOFO. The results for the object percentages and the arithmetic mass of the studied morphological classes are shown in Figure 3. It was found that the maximum number concentration of WHOFO per mass was obtained after 15 min of ultrasonication. The scatter plots and their histograms of results of different ultrasonication durations are shown in Figure S5 in the Supplementary Information. They show a shift to shorter lengths and thinner widths with increasing ultrasonication time that is represented in the calculated geometric mean values. This duration was used in subsequent sedimentation duration variation experiments.

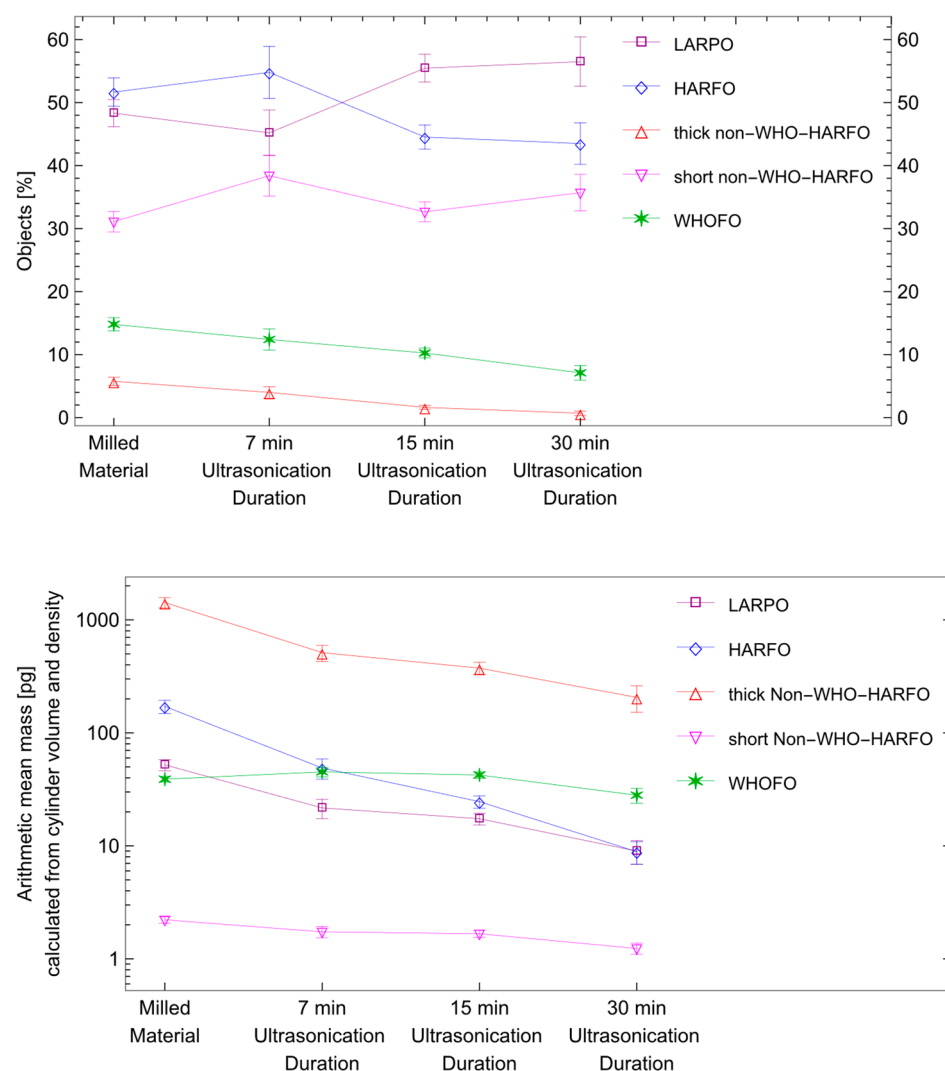


Figure 3. Results from ultrasonication experiments with subsequent sedimentation for 35 min. **Top:** Relative percentage of morphologically classified fragments with respect to the sum of LARPO and HARFO. **Bottom:** Arithmetic mean of the masses of all morphologically classified fragments with measured length and width by assuming they exhibit a cylindrical volume and a specific density of 1.77 g/cm^3 . WHOFO, thick and short non-WHO-HARFO are disjunct sets that make up the HARFO class. The connecting lines are a guide for the eyes only.

Suspension homogenization with an ultrasonic treatment of 15 min improved the reproducibility of the determination of the WHOFO number concentration per mass in Figure 2 and Table 1 and enabled significant increases for prolonged gravitational settling duration. The 4×10^6 WHO-analog fibers per mg material that are required for i.p. testing were achieved after a settling duration of 35 min (group iv).

The number of WHO-analog fibers per mg sample can increase with the settling duration if the total mass of all other objects, i.e., LARPO and non-WHO-HARFO, decreases faster than that of the WHOFO and, at the same time, the number of WHOFO does not decrease significantly faster than that of the other objects. The former is generally expected, as settling is faster for larger objects.

In order to better understand the increase in the number of WHOFO per mg sample during settling, the number of objects per filter area and the process yield in mass per filter area were calculated (cf. Figure 4). As these data sensitively depend on the correctness of the total mass on the filter, the more robust relative measurands object percentage and arithmetic mean of object mass were also calculated and plotted in Figure 5.

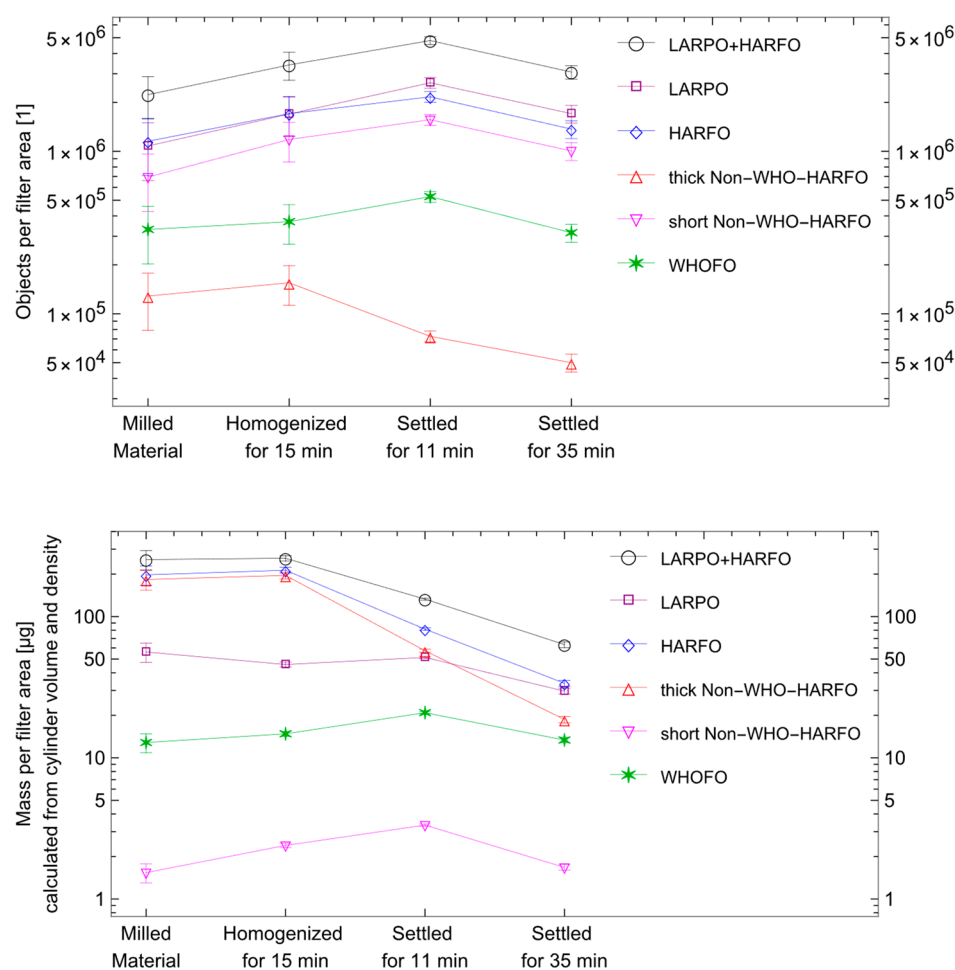


Figure 4. Results from milling, homogenization and settling experiments. **Top:** Total number of morphologically classified fragments per filter and object class. **Bottom:** Total mass per filter and fragments class estimated from the mass of all morphologically classified fragments with measured length and width by assuming they exhibit a cylindrical volume and a specific density of 1.77 g/cm^3 . WHOFO, thick and short non-WHO-HARFO are disjunct sets that make up the HARFO class. The connecting lines are a guide for the eyes only.

The individual object mass was estimated from assuming a simplified cylindrical fragment geometry of length L and diameter W together with a specific CF density of $\rho_{\text{CF}} = 1.77 \text{ g/cm}^3$. Please note that this calculation tended to overrate the true mass in Figure 4, as the maximum mass per filter was $200 \text{ } \mu\text{g}$ (cf. Section 2.2.5). This can be understood by Figure 1, which shows that the majority of fragments exhibited rather an irregular or wedge-like shape than a cylindrical shape.

After homogenization by ultrasonication, the total number of objects per filter area increased as a consequence of the effective separation of agglomerated fragments (Figure 4 top). For a settling duration of 11 min, the number of the largest objects, i.e., the thick non-WHO-HARFO objects, was promptly and significantly reduced. Prolonging the settling time to 35 min also yielded a reduction in the number of all other object classes, including the next-largest particles, the WHOFO.

When analyzing the overall yield of the process as mass per filter (cf. Figure 4 bottom), it becomes evident that the settling procedure reduces the mass yield, and this effect becomes more pronounced with a longer settling duration. It is driven mostly by a reduction in thick non-WHO-HARFO fragments. The necessity of producing an amount of multiple grams in total for the in vivo experiments therefore forced us to limit the settling duration to 35 min, as any prolongation would further reduce the yield of WHO-analog fibers

per experiment. This would have increased the number necessary of production runs to a no longer manageable quantity. About 115 mg of refined material were filtered from 700 mL of supernatant for a single run of the procedure at a settling duration of 35 min. Typically, a minimum of 50 animals per dose level (1×10^9 WHO analog fibers) is required for performing i.p. studies, which results in 147 runs to produce the necessary amount plus reserve material.

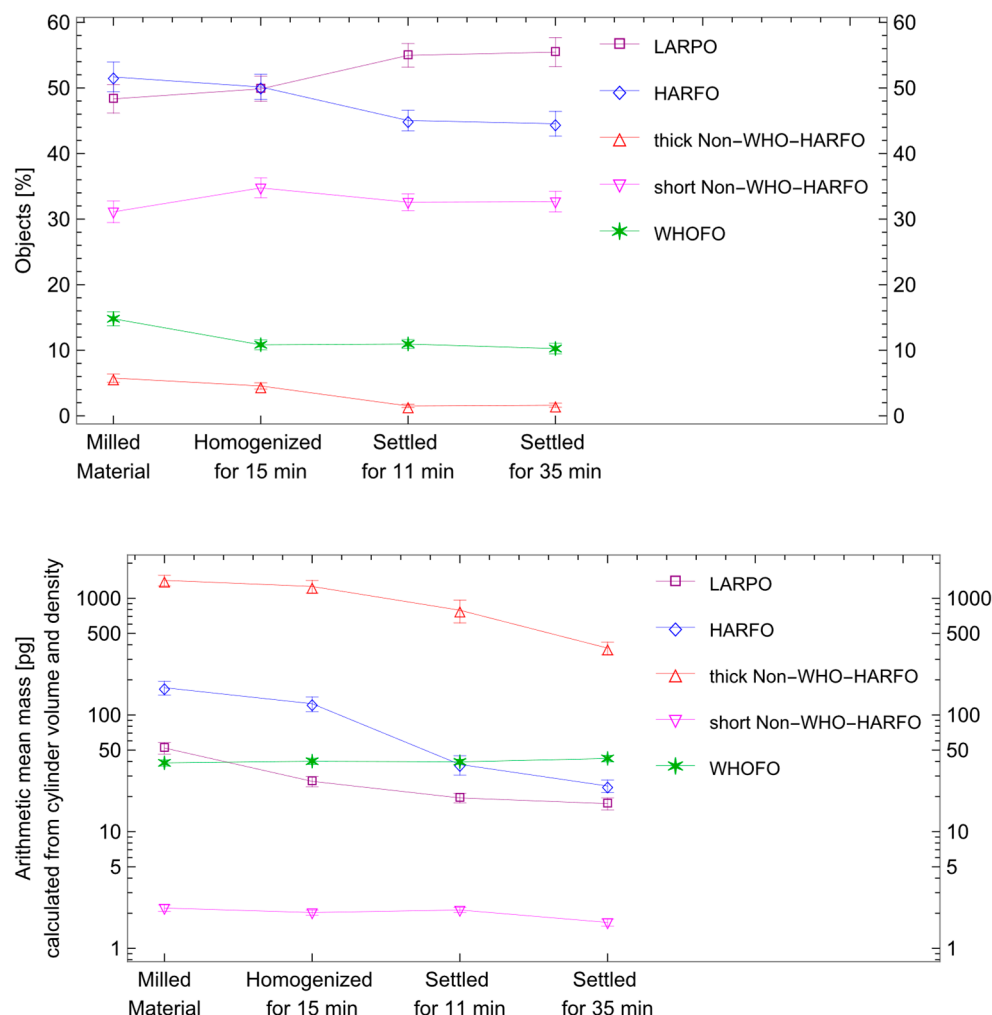


Figure 5. Results from milling, homogenization and settling experiments. **Top:** Relative percentage of morphologically classified fragments with respect to the sum of LARPO and HARFO. **Bottom:** Arithmetic mean of the masses of all morphologically classified fragments with measured length and width by assuming they exhibit a cylindrical volume and a specific density of 1.77 g/cm^3 . WHOFO, thick and short Non-WHO-HARFO are disjunct sets that make up the HARFO class. The connecting lines are a guide for the eyes only.

Figure 5 shows the influence of the process on the relative percentage of morphologically classified fragments (top) and on the arithmetic mean of the masses of all morphologically classified fragments in the cylinder approximation explained above (bottom).

The sum of HARFO and LARPO yields 100%, so any changes in one of the classes are mirrored by the other. It is more instructive to analyze the percentage of the three HARFO subclasses introduced above: The short non-WHO-HARFO make up about 30% of all objects, the WHOFO about 10–15% and the thick non-WHO-HARFO about 1–6%.

In the top of Figure 5, the arithmetic mean masses of the morphological classes are shown. While the arithmetic mean mass for most classes is reduced when introducing and prolonging gravitational settling, this is not the case for the WHOFO. Their arithmetic mean

mass remains constant, which is a consequence of the length and width limits that define the WHOFO and smaller masses of these fragments compared to thick non-WHO-HARFO.

Figure 2 shows the effect of the preparation procedure on the number of WHOFO per sample mass. With the data in Figures 4 and 5, these results can be understood in more detail. The number of WHOFO per sample mass can be calculated from the number of WHOFO objects per filter area (cf. Figure 4, top) divided by the mass of LARPO and HARFO per filter area (cf. Figure 4, bottom). While the number of WHOFO remained constant, the masses attributed to LARPO and HARFO decreased. Thus, there was an increase in the number of WHOFO per sample mass.

During milling, the pre-cut carbon fiber rovings can disintegrate into smaller fragments each time a milling ball hits a fragment. The probability of a fragment being hit and split by a ball is smaller with decreasing fragment size. This results in the observed log-normal distributions for the lengths and widths of HARFO and LARPO [40]. They are shown in Figures 6 and 7 together with a scatter plot of width versus length of all LARPO (purple), HARFO (blue) and WHOFO (gray) from the replicate samples of Table 1. Figure 6 shows the results for group i after milling and suspending but without the sedimentation step, and Figure 7 contains the data from group iv after a settling time of 35 min.

To allow for better orientation, a gray cast and dashed horizontal and vertical lines were overlaid in these figures to denote the WHOFO area. This area is limited by two dashed lines, one at a width of $W = 3 \mu\text{m}$ and the other at a length of $L = 5 \mu\text{m}$. All HARFO to the right of the line at $W = 3 \mu\text{m}$ make up the fraction of thick non-WHO-HARFO, and all HARFO below the line at $L = 5 \mu\text{m}$ make up that of the short non-WHO-HARFO. The effect of different settling durations is visualized by the green, orange and red contour lines in the upper right corner. They correspond to escape diameters D_{esc} of $13.7 \mu\text{m}$, $9.7 \mu\text{m}$ and $6.7 \mu\text{m}$ for volume levels of 700 mL, 350 mL and 175 mL above the residue volume level, respectively. For example, a fragment with an equivalent diameter greater than $9.7 \mu\text{m}$ and an initial position 4.5 cm above the bottom of the vessel can escape into the residue during a settling duration of 35 min. If its initial position lies more than 4.5 cm above the bottom, it will stay in the supernatant. Thus, the effect of fragment size selection varies along the suspension column height.

The dashed diagonal line denotes $W = L$. As the length L is defined as being larger than the width W (cf. Section 2.2.5), no objects are found below this line. In both Figures 6 and 7, the separation between LARPO and HARFO along the line $L/W > 3$ corresponds to the definition of HARFO and LARPO (cf. Section 2.2.5).

The histograms of the length and width distributions correspond to projections of the respective fractions of the scatter plot and were added at the sides. Log-normal distributions were fitted to the data to derive the geometric mean values. Due to the length and width limits that define the WHOFO, their distributions deviate from log-normality. Therefore, log-normal distributions truncated to a length range of $[5, \infty] \mu\text{m}$ and a width range of $[0, 3] \mu\text{m}$ were fitted. In the histogram plots, below the fit results with their error specified, also the calculated geometric mean values (without error) are given. Fitted geometric means that deviated more than three standard deviations from the calculated geometric mean are shown in italics.

Figure 6 shows the size distributions that result directly from the milling process after ultrasonication. It is noteworthy that this process resulted in fragments with a narrow distribution of aspect ratios, i.e., the number of fragments exhibiting an aspect ratio $L/W > 10$ is small. Fractions of thick non-WHO-HARFO and thick LARPO are found in the upper right corner of the central plot, as no gravitational settling was performed. Here a number of fragments is found that exhibit a width of about $6\text{--}7 \mu\text{m}$, which corresponds to the width of the carbon fibers prior to milling. Thus, during the milling process, a small

number of carbon fibers fragmented but remained intact with respect to the diameter. Such fibers are no longer present in Figure 7, as they were efficiently removed by the settling process, together with the majority of the other thick non-WHO-HARFO and thick LARPO.

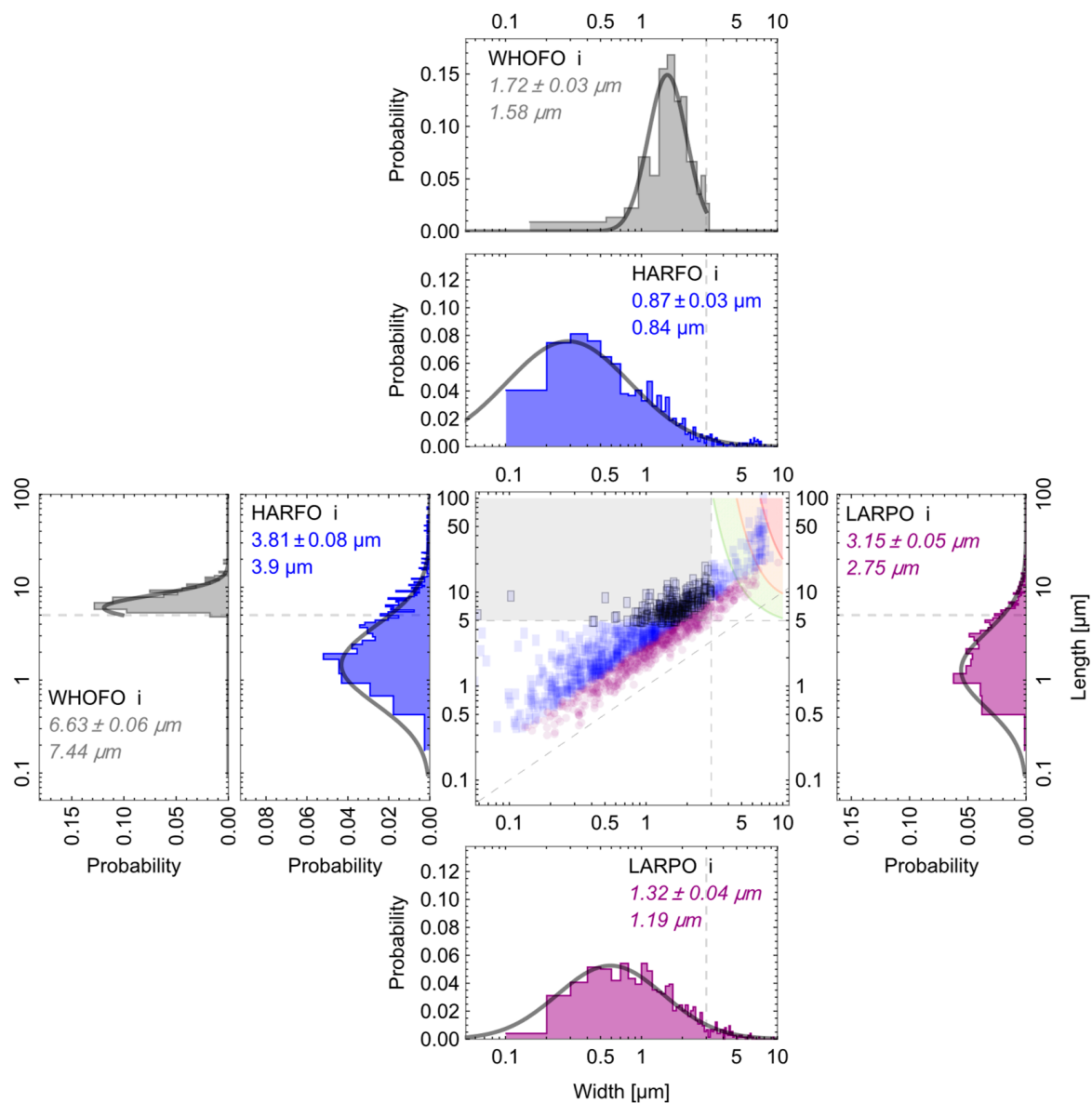


Figure 6. Width vs. length scatter plot and associated probability distribution histograms of LARPO (purple), HARFO (blue) and WHOFO (gray), resulting from projections of the length vs. width scatter plot data, show the replicate samples of Table 1 after milling and suspending (group i). Log-normal distributions were fitted to the data to derive the derived geometric mean values. For WHOFO, log-normal distributions truncated to a length range of $[5, \infty] \mu\text{m}$ and a width range of $[0, 3] \mu\text{m}$ were fitted. The results are specified in the histograms with error, whereas the calculated mean values are specified without error. Fitted geometric means that deviate more than three standard deviations from the calculated geometric mean are shown in italics. The green, orange and red contour lines correspond to escape diameters $D_{esc||}$ of 13.7 μm, 9.7 μm and 6.7 μm for volume levels of 700 mL, 350 mL and 175 mL above the residue volume level, respectively. The overlaid gray cast and dashed horizontal and vertical lines denote the WHOFO area.

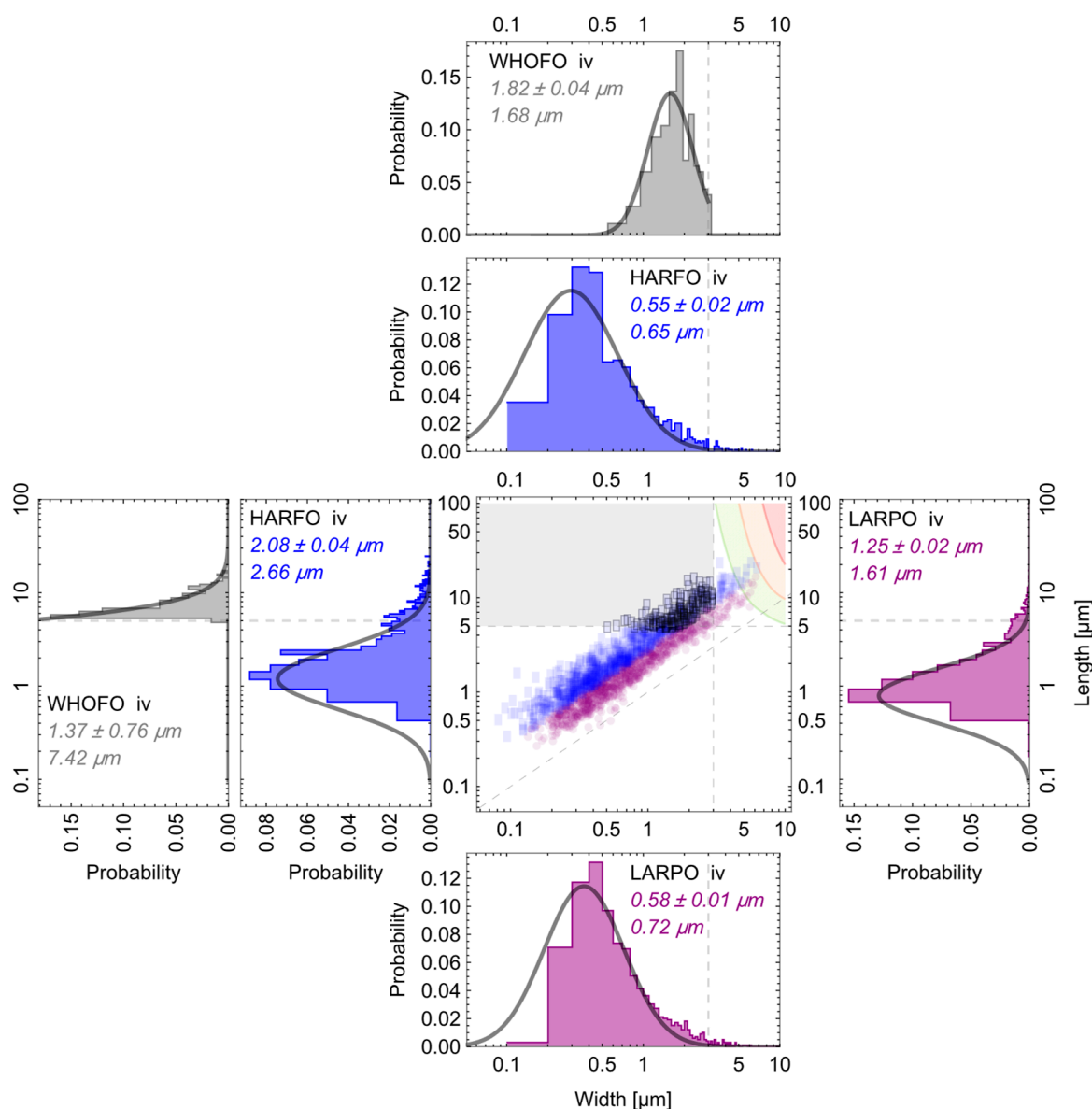


Figure 7. Width vs. length scatter plot and associated probability distribution histograms of LARPO (purple), HARFO (blue) and WHOFO (gray), resulting from projections of the length vs. width scatter plot data, show the replicate samples of Table 1 after 35 min of gravitational settling (group iv). Log-normal distributions were fitted to the data to derive the geometric mean values. For WHOFO, log-normal distributions truncated to a length range of $[5, \infty]$ μm and a width range of $[0, 3]$ μm were fitted. The results are specified in the histograms with error, whereas the calculated mean values are specified without error. Fitted geometric means that deviate more than three standard deviations from the calculated geometric mean are shown in italics. The green, orange and red contour lines correspond to escape diameters D_{esc} of 13.7 μm , 9.7 μm and 6.7 μm for volume levels of 700 mL, 350 mL and 175 mL above the residue volume level, respectively. The overlaid gray cast and dashed horizontal and vertical lines denote the WHOFO area.

Comparing Figures 6 and 7, another effect becomes obvious: the center of gravity of the length and width histograms for HARFO and LARPO shifted to smaller values. At the same time, the geometric mean values in Figures 6 and 7, which are derived either from calculation or from a log-normal fit to the histogram data, start to differ for a settling duration of 35 min in Figure 7. This indicates deviations of the size distributions from a theoretical log-normal shape, which are due to a non-log-normal selection process that

removes larger particles more efficiently from the suspension than smaller ones and thus affects the log-normality of the distributions.

In contrast, comparing the WHOFO objects in Figures 6 and 7, the width and length distribution, constrained by the WHOFO size limits, remain almost unchanged when adding the sedimentation process to the preparation procedure.

It should be noted that the fitting of a truncated lognormal distribution to the tail of the distribution leads to a high uncertainty of the fitting parameters, which is seen, e.g., in the fit of the length distribution of WHOFO in Figure 7. The results from the fitted mean geometric WHOFO length fit were therefore excluded from the data displayed in Figure 8, whereas Figure 9 includes the corresponding calculated geometric mean.

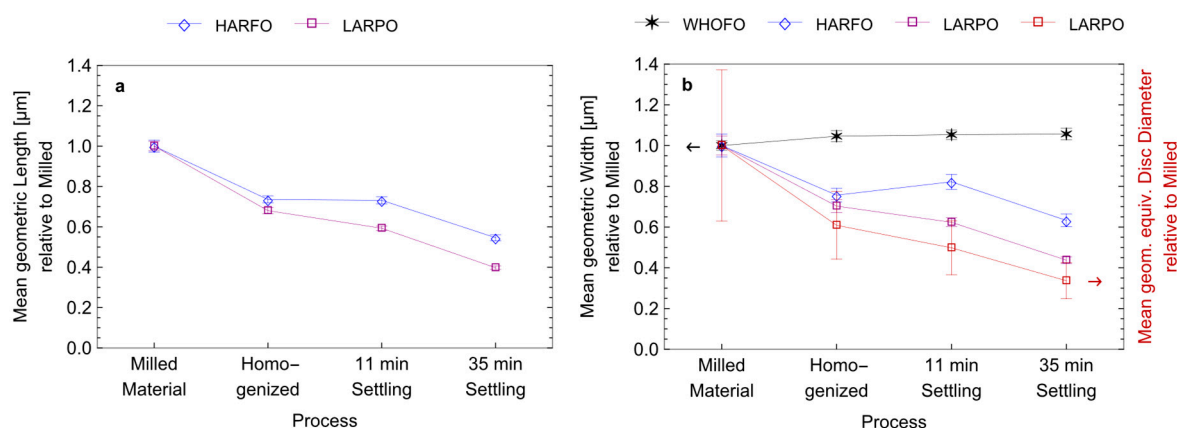


Figure 8. Development of the fitted geometric mean of the lengths (a) and widths (b) normalized to that of the milled and suspended samples for the replicates of the homogenization and sedimentation procedures. The fitted geometric mean values used for normalization to the milled samples were determined as $\langle L_{\text{HARFO}}^{\text{geom.}} \rangle = 3.81 \pm 0.08$, $\langle L_{\text{LARPO}}^{\text{geom.}} \rangle = 3.15 \pm 0.05$, $\langle W_{\text{WHOFO}}^{\text{geom.}} \rangle = 1.72 \pm 0.03$, $\langle W_{\text{HARFO}}^{\text{geom.}} \rangle = 0.87 \pm 0.03$, $\langle W_{\text{LARPO}}^{\text{geom.}} \rangle = 1.32 \pm 0.04$ and $\langle D_{\text{LARPO}}^{\text{geom.}} \rangle = 2.6 \pm 0.7$. The data of the geometric mean WHOFO length are omitted, as they suffer from huge fitting error bars (see Figure 6). A separate y-axis is used for the equivalent disc diameter of LARPO shown in red. The red and black arrows indicate to use the right y-axis for the LARPO data and the left for all other data points, respectively. The connecting lines are a guide for the eyes only.

All these effects are the consequence of the settling process, which systematically reduced the amount of larger, heavier particles while retaining the smaller particles. As the WHOFO belong to the larger and heavier objects after the settling process, their relative contribution to the sample increases as the number of WHOFO per mass increases, and this is the central quality criterion we were aiming to optimize.

The evolution of the fitted mean geometric length and width for HARFO, LARPO and WHOFO over the different processing steps is shown in Figure 8. The corresponding plots for the calculated geometric mean are shown in Figure 9. The data were normalized to the values obtained after milling the sample. A decrease in the mean geometric length and width is observed after homogenization, followed by a further decrease down to 40–60% of the initial value for 35 min of settling duration for the length and width of HARFO and LARPO. This trend and the nearly constant mean geometric width of WHOFO comply with the data trends on mass in Figures 3 and 4. As an object's length and width correspond to its mass, this complies with the rule that heavier particles are more likely to sediment into the residue, whereas smaller particles tended to remain in the supernatant part of the suspension.

The fact that the mean geometric widths of the WHOFO remained almost constant is a consequence of the fact that WHOFO are, by definition, confined to widths below 3 μm. This size range was little affected by the selective removal of larger fragments, as

the gray-shaded WHOFO area does not overlap with the region marked for three different escape diameters $D_{\text{esc||}}$ in Figure 7.

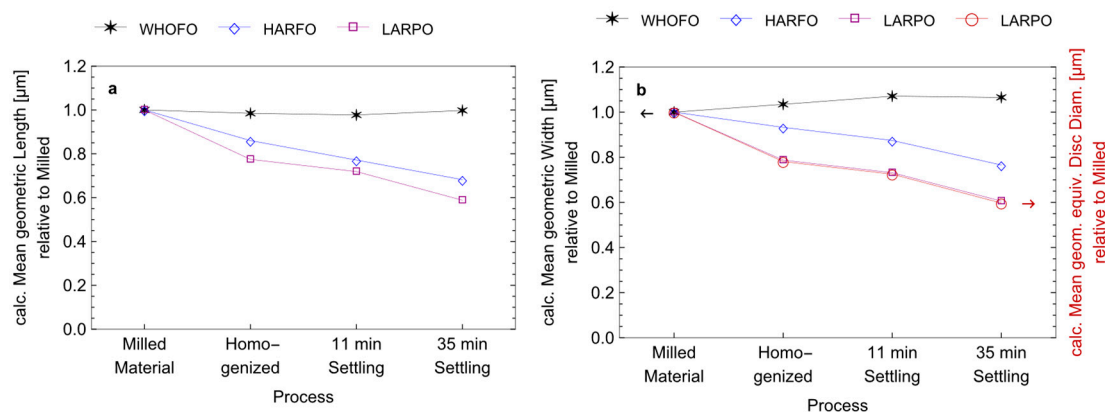


Figure 9. Development of the calculated geometric mean of the lengths (a) and widths (b) normalized to that of the milled and suspended samples for the replicates of the homogenization and sedimentation procedures. The geometric mean values used for normalization of the milled samples were calculated as $\langle L_{\text{WHOFO}}^{\text{geom.}} \rangle = 7.44$, $\langle L_{\text{HARFO}}^{\text{geom.}} \rangle = 3.90$, $\langle L_{\text{LARPO}}^{\text{geom.}} \rangle = 2.75$, $\langle W_{\text{WHOFO}}^{\text{geom.}} \rangle = 1.58$, $\langle W_{\text{HARFO}}^{\text{geom.}} \rangle = 0.84$, $\langle W_{\text{LARPO}}^{\text{geom.}} \rangle = 1.18$ and $\langle D_{\text{LARPO}}^{\text{geom.}} \rangle = 2.04$. A separate y-axis is used for the equivalent disc diameter of LARPO shown in red. The red and black arrows indicate to use the right y-axis for the LARPO data and the left for all other data points, respectively. The connecting lines are a guide for the eyes only.

As settling affects larger fragments stronger than smaller ones, the separation effect can be visualized determining the 95th percentiles of the width and length distributions for the three types of fragments in Figure 10. For the milled material, the 95th percentile of the length distribution of HARFO was about 25 m. Additional homogenization and settling steps reduced it to about 11 m. A similar but less pronounced trend was found for the equivalent disc diameter of LARPO, whereas the 95th percentile of WHOFO width distribution remained constant. Although decreasing with the settling duration, Figure 10 shows that, even after 35 min, the 95th percentile of the LARPO equivalent disc diameter distribution is still a bit above the value of $D_{\text{LARPO}}^{95\text{th}} = 4 \mu\text{m}$ that is recommended for i.p. testing in rodents.

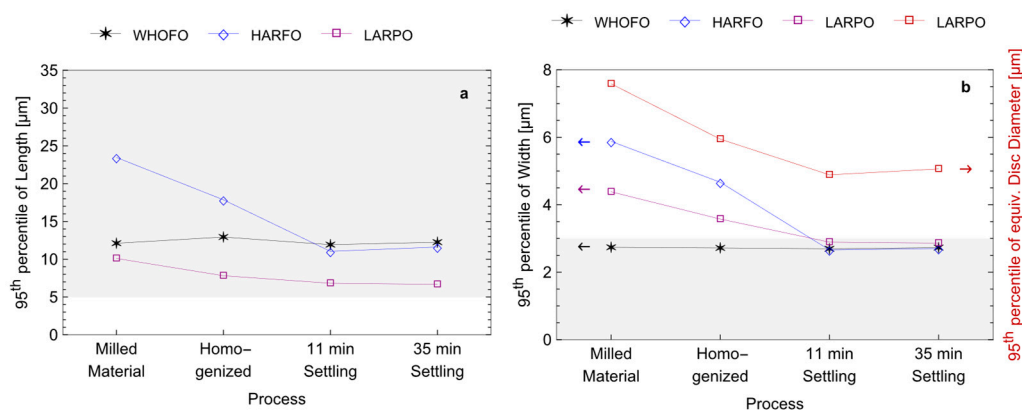


Figure 10. Development of the 95th percentile of the lengths (a) and widths (b) for the replicates of the milling, homogenization and sedimentation procedures. For LARPO, an equivalent disc diameter was determined. The gray cast marks the targeted length and width ranges that are characteristic for WHOFO. A separate y-axis is used for the equivalent disc diameter of LARPO shown in red. The arrows indicate to use the right y-axis for the LARPO data and the left for all other data points, respectively. The connecting lines are a guide for the eyes only.

4. Discussion

Details of the milling process are crucial for the resulting disintegration of the studied CF material. In general, brittle materials can disintegrate into respirable fragments due to unidirectional or fiber axis-directed impactation or shear energy transfer. Likewise, intrinsically anisotropic materials were found to disintegrate into fibrous constituents or fragments during, e.g., planetary ball milling of CF [11] or ball milling of bamboo or jute fibers for the preparation of nanocellulose [41,42]. Other milling techniques used in cutting or rotor mills were not considered suitable for our purpose of toxicological testing, as they focus on length cutting. Since ball milling causes different mechanical failure modes in a material by both direct and indirect, as well as unordered multidirectional impactation and abrasion, it can reveal the general propensity of a material to form fibrous fragments [34]. For fibers of initial diameters exceeding the minimum WHO length requirement of 5 μm , as is standard for CF, transversal fragmentation can, in principle, result in fragments of critical geometry, whereas directional disintegration can originate from or be promoted by intrinsic anisotropic microstructural characteristics of the carbon material. As repeated impactation and sequential splitting of larger fragments into smaller ones tend to form the observed log-normal-like size distributions, it takes continuously increasing milling durations to shift the geometric mean sizes of a milled batch to smaller values [43]. Compared to a granular fragment of the same diameter, a fiber-shaped fragment being milled requires several impacts to form granular fragments. It is therefore principally possible for fibers to survive a milling process for some time, provided the geometric mean size of the milled batch is not excessively reduced to a level that no longer contains the WHO-analog fiber fragments of interest. Indeed, ball milling of PAN-based CF was observed here to form fibrous fragments that exhibited such WHO-analog dimensions.

After an initial de-agglomeration step using ultrasonication, gravitational settling in liquid suspension was studied in order to enrich the number of WHO-analog fibers with respect to the total fragment mass. Discussions on the selection of form factors to properly describe the settling of spherical, elongated or irregularly shaped particles have recently gained new momentum from the microplastics research community [33,38,44,45]. The authors of Ref. [44], for instance, predicted only weak effects of shape on the settling velocity of carbonaceous particles with equivalent diameters below 6.3 mm in 2-propanol, which is not compatible with the observations in Ref. [38]. Similarly, the authors of Ref. [33] observed perpendicular settling and predicted the long-range transport of microscale fibers. However, the irregular or wedge-like HARFO shapes of our carbon fiber fragments suggest that they could tend to orientate randomly (tumble) or vertically (parallel) to the settling direction and thus exhibit a larger effective equivalent diameter than a LARPO of the same mass. Stabilized perpendicular settling, as was observed in Refs. [33,38] for microscale fibers of close-to-perfect cylindrical shape, is considered less likely for our fragments.

Experimentally, the gravitational settling of our HARFO fragments was observed to proceed faster in general than that of the LARPO fraction and is therefore, in principle, not in favor of enriching fibers. However, with respect to all fragments, the fraction of WHO-analog fiber objects was nonetheless enriched both in absolute number and in number per mass. This results from the fact that WHOFO are, by definition, confined to widths below 3 μm . That size range was little affected by the selective removal of larger fragments, as the gray-shaded WHOFO area in Figure 7 does not overlap with the region marked for the three different escape diameters $D_{\text{esc}||}$. Therefore, the depletion of HARFO and LARPO fragments exceeding this width limit of 3 μm occurred faster than that of WHOFO.

By combined optimization of the milling and gravitational suspension settling conditions, it was thus possible to provide a material that showed a 15-fold increase from 0.36×10^6 to 6×10^6 WHO-analog fibers per sample mass compared to the milled CF

material and complied with the regulations for i.p. application, which restricted the total injected test material mass (see Table 1).

The observed trends in the geometric means of length, width or equivalent disc diameter resulting from the process parameter variations are considered statistically significant, especially the relative enrichment factor of about 15. Statistical ensemble errors and error propagation effects were calculated and considered during error-weighted data fitting. The question of the validity of the assumption of a log-normal shape of the length and width distributions was addressed by comparing the calculated and fitted geometric means of the distributions. The fitting of the truncated distributions of the WHOFO lengths was of limited prediction reliability, however, for the geometric mean of the untruncated HARFO distribution, since its mean was close to or below the WHOFO length limit of 5 μm and therefore not covered with populated bins. Likewise, the calculated geometric mean of a truncated log-normal distribution can never predict or represent the geometric mean of the untruncated distribution.

From an occupational safety perspective, it is beneficial to separate CF fragments in a liquid but not by aerosolization methods. The latter can be a source of respirable hazardous particles and thus be harmful to the operator [31,32,46]. Aerosolization sources are, however, appropriate for toxicological studies on inhalative applications in closed aerosolization and exposure set-ups.

2-propanol was chosen as a suspension liquid of manageable hazard properties with respect to flammability, irritability and environmental concern by disposal. Compared to water, 2-propanol exhibits a lower density and a higher viscosity, resulting in about 1.8 slower sedimentation. Water, a less toxic liquid, even of ultra-filtrated quality, tends to contain many micro- and nanoscale particles, whereas the 2-propanol quality used for this work dried without residue. To obtain ultrapure water, free of micro- and nanoscale particles, it is, however, recommended to prepare freshly double-distilled water [47]. It is also important to consider that more polar solvents like water can promote fragment agglomeration of non-polar materials like carbon or polymers [48]. However, as an organic solvent, 2-propanol could dissolve carbon fiber sizings and recoat the fragment surface upon drying. High-resolution SEM images, however, showed no indications of such a coating.

The parameter range studied here for optimizing the WHO-analog fiber yield and concentration was limited by the time required to obtain statistically representative particle size distributions for each experiment, as it requires the tedious and time-consuming morphological evaluation of many collected fragments on numerous SEM images.

With the help of our semi-automated image evaluation workflow, it was possible to identify a process chain that led to one order of magnitude of relative enrichment. This enabled us to provide sufficient amounts of a test material that conforms with the requirements of toxicological i.p. animal testing with a still manageable material preparation effort. The main deficit of the binary semantic segmentation used for semi-automated image evaluation was that overlapping fragments were not separated automatically, i.e., recognized as separate particle instances. Therefore, all images had to be visually checked for correctness and completeness, and the segmentation was amended whenever necessary. Despite this additional workload, the image segmentation was extremely accelerated, and optimum quality, i.e., on par with human instance segmentation, was obtained. Fully automated evaluation using advanced artificial neural networks is expected to enable investigating a significantly larger parameter range within a comparable amount of time and workload in the future.

5. Conclusions

Earlier statements [49–52] that carbon fibers do not form respirable fiber dusts and are therefore intrinsically safe per se were not confirmed by recent studies [34,53,54]. This raised occupational health concerns with regards to inhalation exposure to carbon fiber dusts and motivated fiber toxicological studies on carbon fiber fragments [17].

In order to avoid so-called overload effects caused by low-soluble, low-toxicity dust particles for fiber toxicological assessment by intraperitoneal tests of fragments formed from solid materials, the disintegrated material must be applied with a sufficiently high proportion of fragments of concern. To satisfy this constraint, the present study enriched the WHO-analog fiber-shaped fragments in samples of ground PAN-based CF with respect to other fragments.

This was accomplished by the systematic optimization of the conditions for milling, suspension homogenization, liquid-phase gravitational settling and collection of the resulting supernatant. The procedure described here combined ball milling, liquid suspension, ultrasonication and gravitational settling in a process that removed large fragments more effectively from the supernatant than WHO-analog fibers. By careful morphological characterization of the obtained test material, the process was shown to be capable of increasing the concentration of WHO-analog fibers per mass of ground polyacrylonitrile-based carbon fibers by a factor of about 15.

It was shown that the fiber concentration of the enriched material reached a level that is suited for intraperitoneal testing in rodents. The present work therefore enabled us to schedule a toxicological study with intraperitoneal syringe application of the obtained material. This will require drying and re-dispersion of the fiber-enriched material in a suited injection medium [55].

Intraperitoneal testing in rats is accepted by EU regulatory authorities as a way to investigate the carcinogenic potential of biopersistent fibers. Therefore, the results of subsequent toxicological studies using our enrichment approach are expected to have high regulatory relevance, as they provide scientific data required for the development and implementation of a coherent regulatory framework for critical fibers.

Time- and cost-efficient but a nonetheless detailed and statistically representative morphological characterization of particle- and/or fiber-containing material samples is of central importance not only for material enrichment process development but also for toxicological studies and occupational fiber exposure compliance testing [56]. The latter two aim at assessing health risks related to quantified biopersistent respirable fiber fractions in dusts emitted, e.g., during the production, use and recycling of advanced materials like carbon nanotubes, carbon nanofibers, carbon fibers and carbon–carbon composites.

To reliably assess the material quality, traditionally, a tedious and time-consuming morphological characterization of the fragments formed is mandatory. This task was facilitated and greatly accelerated by the use of semi-automated software tools and artificial neural networks developed in-house. Further progress in reliable automated characterization of sampled particle ensembles appears desirable nonetheless.

Artificial neural networks capable of so-called instance segmentation, i.e., recognizing adjacent or overlapping particles as individual objects, are expected to further improve the morphological quality in the future. The SEM images that were instance-annotated by computer-assisted humans in this work could provide the so-called ground truth data required for training such networks.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fib13060069/s1>: Figure S1: Application of the counting rules: (a) An object with evenly cut end protruding outside the image frame is taken into account. (b) Particle cluster

consisting of distinguishable individual fragments are counted separately, here 3 objects in black, grey and green. (c) Objects which are in contact with or even align to a larger object. The blue object is not taken into account when counting objects according to the counting rules, whereas the purple, red and green objects are to be counted as well as the black object.; Figure S2. Ultrasonication of the suspension of milled CF and 2-propanol.; Figure S3. Different stages of the evaluation of SEM images: (a) SEM-Image as acquired. A purple frame marks the zoomed-in-area used for (b); (b) selected object(s) zoomed-in prior to annotation; (c) semantic binary segmentation mask after ANN processing: recognized objects appear in dark blue. A red frame marks the zoomed-in-area used for (d); (d) annotated objects labelled A, B and C after use of ANN for annotation appearing in blue; (e) segmentation mask after manual reprocessing: objects after manual modification appear in green or purple for objects that protude out of the image. A yellow frame marks the zoomed-in-area used for (f); (f) several objects in green after manual reprocessing following the counting rules and added labelling for the distinguished segments 1–4. The blue object B remains unchanged from the ANN annotation since no changes were necessary. Impurity segment C (as visible in image (d)) was deleted.; Figure S4. Width and length probability distributions histograms of LARPO (purple), HARFO (blue) and WHOFO (gray), being the projections of the length vs. width scatter plot data, show the replicate samples of Table 1 after 15 min of ultrasonic treatment (group ii) and after additional 11 min of gravitational settling (group iii). For WHOFO, log-normal distributions truncated to a length range of $[5, \infty]$ μm and a width range of $[0, 3]$ μm were fitted. Fitted geometric means that deviate more than three standard deviations from the calculated geometric mean are shown in italics. The green, orange and red contour lines correspond to escape diameters d_{escape} of 13.7 μm , 9.7 μm and 6.7 μm for volume levels of 700 mL, 350 mL and 175 mL above the residue volume level, respectively. The overlaid gray cast and dashed horizontal and vertical lines denote the WHOFO area.; Figure S5. Width and length probability distributions histograms of LARPO (purple), HARFO (blue) and WHOFO (gray), being the projections of the length vs. width scatter plot data, show the replicate samples after 7 min (a), 15 min (b) and 30 min (c) of ultrasonic treatment and 35 min of gravitational settling. For WHOFO, log-normal distributions truncated to a length range of $[5, \infty]$ μm and a width range of $[0, 3]$ μm were fitted. Fitted geometric means that deviate more than three standard deviations from the calculated geometric mean are shown in italics. The green, orange and red contour lines correspond to escape diameters d_{escape} of 13.7 μm , 9.7 μm and 6.7 μm for volume levels of 700 mL, 350 mL and 175 mL above the residue volume level, respectively. The overlaid gray cast and dashed horizontal and vertical lines denote the WHOFO area.; Figure S6. Probability distributions of the volumes of assumed cylindrical fragments of the measured length and width of LARPO (a), HARFO (b) and WHOFO (c) for the replicate samples of Table 1 after milling and suspending (i), followed by ultrasonication (ii) and after 11 min (iii) or 35 min (iv) gravitational settling. The WHOFO distribution was fitted with the PDF of a log-normal distribution. The corresponding mass can be estimated by multiplying the volume with the CF density of 1.77 $\text{pg}/\mu\text{m}^3$.; Figure S7. Aspect ratio (L/D) probability distributions of LARPO (a), HARFO (b) and WHOFO (c) for the replicate samples of Table 1 after milling and suspending (i), followed by ultrasonication (ii) and after 11 min (iii) or 35 min (iv) gravitational settling. The ratio of the PDFs of the log-normal distributions for length and width, truncated to the according aspect ratio ranges, were fitted to predict the difference $\mu_L - \mu_W$ of the geometric means of the length and the width distributions.

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Data Availability Statement: Numerical data on the fragment morphology of the ultrasonication and the settling series used for this work are provided in JSON format online. Other data are available from the corresponding author upon request.

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