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## Experimental approaches to data generation for REACH compliance of multi-walled carbon nanotubes: environmental fate

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Applying regulatory-accepted, standardised test guidelines to carbon-based nanomaterials is challenging. A primary difficulty is to analytically distinguish these nanomaterials from the high carbon background of the environment. Further challenges arise from the physicochemical characteristics of multi-walled carbon nanotubes (MWCNTs), which form entangled, highly cohesive bundles that agglomerate rapidly. As a result, many analytical methods are not suitable. This study evaluated the applicability of existing standardised analytical methods and explored potential alternatives. We focused on EU-REACH data requirements related to environmental fate, specifically nanomaterial dissolution, dispersion stability, and adsorption/desorption properties. Additionally, we assessed the feasibility of a sewage treatment plant (STP) simulation study according to OECD TG 303A, considering various analytical methods, including isotopic signatures. The findings of this study highlight challenges and novel analytical approaches in generating data for REACH registration purposes, and identify research needs.

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### Environmental significance

Generating experimental data for the environmental fate assessment of carbon-based nanomaterials such as multi-walled carbon nanotubes (MWCNTs) under the REACH regulation remains challenging due to the lack of applicable and standardised analytical methods. Our research focusses on the feasibility of applying both existing and novel analytical techniques to generate environmental fate data for MWCNTs. Alongside nanomaterial dissolution and dispersion stability, we assessed the viability of a sewage treatment plant (STP) simulation study, using advanced methods such as isotopic signatures and Raman spectroscopy. Given the increasing production and release of nanomaterials into the environment, our work addresses the urgent need for analytical methods to perform reliable and robust chemical risk assessments.

## Introduction

In the European Union, manufactured or imported chemicals must be registered according to the provisions of the REACH regulation (Regulation (EC) No 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals). REACH annexes VI to X specify the standard information requirements for registration purposes. In 2018, additional nano-specific data requirements were published for these

annexes, implemented in Commission Regulation (EC) 2018/1881. Since January 2020, registrants of nanomaterials have been obliged to provide nano-specific information for compliant REACH registrations. According to Article 13(3) of the REACH regulation, tests conducted to fulfil these information requirements ‘shall be conducted in accordance with the test methods laid down in a Commission Regulation or in accordance with other international test methods recognised by the Commission or the Agency as being appropriate’. Based on this provision, newly generated experimental data for REACH registration purposes are generally accepted only if standardised and validated test guidelines are followed, as defined in the EU test methods regulation (Commission Regulation (EC) No 440/2008), which refers to test guidelines (TGs) of the Organisation for Economic Co-operation and Development (OECD) for many of the REACH-required tests. To date, nano-specific test guidelines are often missing or have only a limited applicability,<sup>1–3</sup> thus impeding the

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production of reliably accepted data. The situation is particularly challenging for purely carbonaceous nanomaterials, such as MWCNTs.

Relevant Guidance Documents of the European Chemicals Agency (ECHA) address carbonaceous nanomaterials only to a limited extent and provide no guidance on how to consider the specific properties of MWCNTs, which cause severe problems in conducting tests required by REACH. These properties include extensive aggregation/agglomeration that leads to highly unstable dispersions and analytical problems in carbon-containing media.<sup>4–8</sup> It is therefore not surprising that authorities recently published calls for identifying information gaps related to nanomaterials. For example, in 2024 and 2025, ECHA's European Union Observatory for Nanomaterials (EUON) issued calls for 'closing nanomaterials' information gaps: EUON welcomes new study proposals' and 'EUON calls for study proposals to address nanomaterials knowledge gaps in the EU'.<sup>9,10</sup>

Due to the limited availability of standardised and validated test guidelines for generating the obligatory nano-specific experimental information, registrants currently face several complex challenges. First, testing has to be adapted to some extent to address the properties of the specific nanomaterial (*e.g.*, poor dispersion stability) but guidance for such adaptations is often lacking for the specific TG that is requested by legal provisions. Second, while there is a wealth of academic literature on testing nanomaterials using a variety of approaches, their regulatory acceptance is often unclear to registrants. For example, a recent ECHA-sponsored report on existing methods for the detection and quantification of carbon-based nanomaterials in environmental and biological matrices<sup>11</sup> lists 57 methods and analytical techniques. This report also notes the lack of standardised protocols, making it uncertain whether 'resulting data are already accepted, or could soon be accepted, by authorities, and ECHA in particular, under legislation such as REACH'.<sup>11</sup> This finding illustrates (a) the problems registrants face when selecting appropriate quantification techniques for studies required by REACH and (b) the uncertainty they have with respect to regulatory acceptance of their data. Third, registrants typically commission contract research organisations (CROs) with expertise in applying a specific TG. However, a CRO's experience in applying that method specifically to a nanomaterial (in this case MWCNTs) may be limited. For (eco)toxicological tests, REACH Article 13(4) further specifies that a CRO must be accredited according to principles of Good Laboratory Practice (GLP), further limiting the number of CROs that can conduct the required test. In addition, a CRO may not have all analytical techniques available that could be envisaged. Since all techniques have their advantages and disadvantages for carbon-based nanomaterials, a 'multi-technique approach is often necessary',<sup>11</sup> making it even more unlikely that a CRO with a specific OECD TG expertise will be equipped with all possible methods. Finally, generating data for REACH registration purposes often has to be performed within a certain time

period due to legal deadlines and within a given budget, limiting the possibilities of applying a variety of different test conditions (*e.g.*, extending those prescribed in OECD TGs) or analytical techniques. The issues discussed above are interlinked. For example, a registrant is unlikely to spend extensive resources on several analytical techniques if their regulatory acceptance is questionable.

This study aims to share the knowledge gained through the practical application of TGs to fulfil REACH information requirements. It showcases the technical and practical challenges of generating data for real REACH registration dossiers, highlighting failures and successes that we encountered while generating new data, along with feasible adaptations to test guidelines that we believe are acceptable to regulatory bodies. It provides a realistic regulatory perspective that differs from studies conducted by research institutes or academia in several respects. For example, following the required TGs means that test concentrations in most cases cannot be chosen freely. Also, applying different analytical techniques is limited by availability as well as budget and time constraints, as discussed above. These constraints generally also prevent the repetition of studies under refined conditions after more experience has been gained. While these issues may be perceived as limitation, this study provides real-world insights that are of interest for stakeholders involved in data generation for REACH registration purposes and the broader scientific community interested in test methods for carbon-based nanomaterials.

As is evident from the above, this study is not a review of the existing literature for testing a specific endpoint, does not intend to validate TGs or analytical techniques for MWCNTs, nor does it aim to define new standard procedures; instead, it introduces innovative experimental approaches to overcome challenges of generating data for specific, difficult-to-assess endpoints of MWCNTs in a REACH context.

While some progress has been made in developing standardised test methods for environmental fate endpoints to fulfil REACH requirements for nanomaterials,<sup>12,13</sup> challenges remain for some endpoints and for MWCNTs in particular. This paper focuses on such environmental fate endpoints under REACH annexes VII and VIII, for which novel analytical methods are explored to overcome potential non-compliance issues. These endpoints include nanomaterial dissolution, dispersion stability, and adsorption/desorption properties. Challenges of data requirements related to substance identification and human health are addressed in the companion articles.<sup>14,15</sup>

### Water solubility and dissolution rate according to REACH annex VII, section 7.7

Carbon is ubiquitously present in the environment. Therefore, it is extremely difficult to measure carbon release into aqueous media from carbon-based nanomaterials, such as graphene, single-walled carbon nanotubes (SWCNTs) or



MWCNTs. In addition, carbon can be released from experimental equipment, further complicating the measurement of carbon dissolution from carbon-based nanomaterials. Elemental carbon appears to be insoluble in almost every solvent because of, *inter alia*, its very stable structure and chemical bonds, its lack of polarity and functional groups. Yet, investigating water solubility and/or dissolution of nanomaterials is a data requirement under the REACH regulation. In order to comply with regulatory requirements, we investigated the carbon water solubility of MWCNTs—or more accurately, the dissolution of carbon into water—to a technically feasible extent. Although the problem of determining the dissolution of carbon into aqueous, naturally carbon-containing environmental matrices is noted, the Guidance Document (GD) OECD No. 318 (ref. 4) does not contain specific advice on how to address this issue. We developed a new approach combining OECD Test Guideline (TG) 105 (ref. 16) and OECD GD 318 (ref. 4) to monitor carbon dissolution *via* a conventional column elution method, while excluding any external carbon source. This was achieved by using glassware only, as well as sand and glass wool as alternative column filling material.

#### Partition coefficient *n*-octanol/water or dispersion stability according to REACH annex VII, section 7.8

The partition coefficient *n*-octanol/water ( $K_{ow}$ ) is generally used as a physicochemical parameter to provide information on a chemical's lipophilicity and, more broadly, on the tendency of a chemical to bioaccumulate and adsorb to solids. However, the concept of  $K_{ow}$  does not apply to inorganic substances and often to nanomaterials.<sup>17</sup> In cases where  $\log K_{ow}$  does not apply, the REACH regulation instructs the registrant of both organic and inorganic nanoforms to investigate the dispersion stability in environmental media instead. The corresponding standard test guideline is OECD TG 318.<sup>18</sup> We conducted OECD TG 318 with MWCNTs, aiming to produce meaningful data despite anticipating that MWCNTs would be hardly dispersible.

#### Degradation and adsorption/desorption screening according to REACH annex VIII, sections 9.2 and 9.3

MWCNTs are defined as an inorganic substance and have been determined to be neither soluble in water nor octanol. For inorganic substances, biodegradation screening tests can be omitted according to REACH annex VII. However, there are no waiving options for inorganic (insoluble) nanomaterials regarding simulation testing in water, sediment, and soil. Rather, according to REACH annex IX, transformation and abiotic and biotic degradation products must be identified. Furthermore, according to annex VIII of REACH, tests for insoluble nanoforms must specifically consider morphological transformation, chemical transformation, and other abiotic degradation. Finally, it is not possible to waive data for stable nanomaterials based on

physicochemical properties alone, unless its relevance for a low potential for adsorption can be adequately justified. As there is currently no simple relationship identified between a single physicochemical property and the adsorption behaviour of nanomaterials, such a justification is precluded, and experimental data are required.

Based on known physicochemical properties of MWCNTs, they are expected to distribute to the sewage sludge solids fraction. We therefore focussed on generating viable data on the distribution of MWCNTs in sewage treatment plants (STPs). For example, concerning higher tier simulation tests for degradation, OECD TG 303A 'Simulation Test – Aerobic Sewage Treatment – Activated Sludge Units'<sup>19</sup> is mentioned in the ECHA Guidance on nanomaterials as '*useful, in particular for assessing the distribution of nanoparticles in sewage treatment plants.*'<sup>6</sup> Given the significantly higher test material concentration applied in OECD TG 303A compared to other degradation simulation test guidelines, this was the only test guideline potentially capable of producing relevant results: only high test material concentrations may allow the determination and quantification of MWCNTs against the high carbon background of environmental media.<sup>8</sup> Importantly, an OECD TG 303A study could not only provide information on any morphological changes of the nanomaterial while passing through the sewage treatment system *via* visual investigations of microscopic images, but also yield an apparent adsorption coefficient determined from the extent of adsorption to sewage sludge solids. This could fulfil the requirement for further investigations on the degradation of insoluble nanoforms according to REACH annex VIII, section 9.2, and at the same time satisfy information requirements on adsorption/desorption. Here, we report on a feasibility study for assessing MWCNTs' environmental fate according to OECD TG 303A and our investigations into the applicability of various analytical methods to track and quantify carbonaceous nanomaterials in the environment. While OECD TG 303A was developed to assess biodegradation of water-soluble organic substances,<sup>19</sup> it was considered meaningful for the reasons discussed above.

## Materials

### Multi-walled carbon nanotubes substance-specific information

**K-Nanos.** K-Nanos MWCNTs are elongated high-aspect-ratio MWCNTs that are aligned and entangled in bundle-type carbon agglomerates. Here, K-Nanos comprised three nanoforms, which were considered a 'set of similar nanoforms' based on REACH annex VI, amended by Commission Regulation (EC) 2018/1881: K-Nanos 100, K-Nanos 210, and K-Nanos 300. The purity of K-Nanos was >91% (SI Table S1). The characteristics of constituent particles are as follows: the average individual carbon nanotube length and diameter ranged from 46.22–62.76  $\mu\text{m}$  and 12.13–14.58 nm, respectively. Derived from these values,



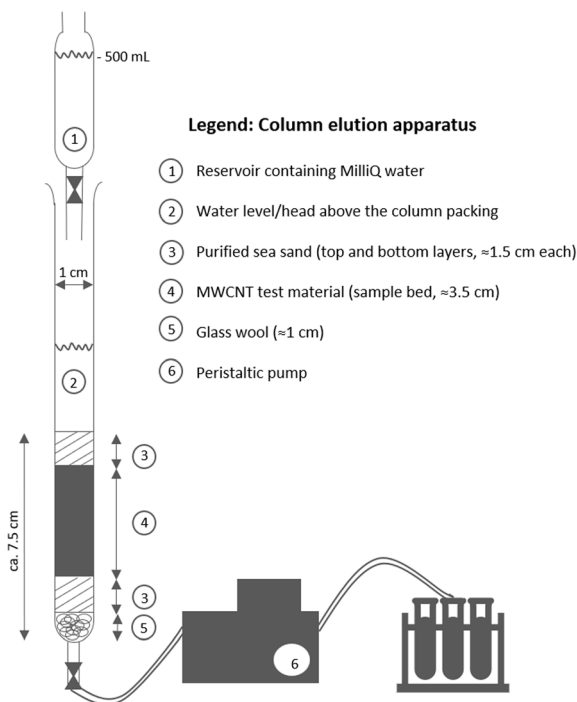
the aspect ratio was 3789:1–4304:1. Average bundle length of MWCNTs ranged from 42.00–54.85  $\mu\text{m}$ , while average bundle diameter ranged from 2.46–3.21  $\mu\text{m}$ . The specific surface area and the density at 20 °C were 200.8–247.7  $\text{m}^2 \text{g}^{-1}$  and 1.76  $\text{g cm}^{-3}$ , respectively. Nanoform-specific data can be found in SI Table S1.

**Jenotubes.** Similarly, Jenotube MWCNTs are elongated high-aspect-ratio MWCNTs that are aligned and entangled in bundle-type carbon agglomerates. Here, Jenotubes comprised four nanoforms, which were considered as a ‘set of similar nanoforms’: Jenotube 6, Jenotube 8, Jenotube 10A, and Jenotube 10B. The purity of Jenotubes was >97% (SI Table S2). The characteristics of constituent particles are as follows: average individual tube length and diameter ranged from 35.87–136.37  $\mu\text{m}$  and 7.4–12.2 nm, respectively. Based on these values, the aspect ratio was calculated as 3261:1–16 961:1. Average bundle length of MWCNTs ranged from 35.6–129.8  $\mu\text{m}$ , while average bundle diameter was in the range of 2.9–6.3  $\mu\text{m}$ . Lastly, the surface area and the density at 20 °C were 217–644  $\text{m}^2 \text{g}^{-1}$  and 2.29  $\text{g cm}^{-3}$ , respectively. Nanoform-specific data can be found in SI Table S2.

## Methods

### Water solubility or dissolution of nanomaterial

Determination of the water solubility (carbon dissolution) from MWCNTs was performed with K-Nanos and Jenotubes using a column elution method according to OECD TG 105 (ref. 16) and OECD GD 318 (ref. 4) (section 2.2 ‘dynamic testing of dissolution rates’). The study was conducted in ultrapure water containing <200  $\mu\text{g C L}^{-1}$  background concentration, measured as non-purgeable organic carbon (NPOC). The glass column with fused glass frit (see SI for details) and all accessory materials were selected to minimise any potential external carbon contamination to the extent technically feasible. For example, Pt-cured silicone tubing (1.0 mm inner diameter, 2.84 mm outer diameter) was used for connecting the column with the peristaltic pump and the pump with the all-glass collection vials. The narrow column (15 mL) with frit and stopcock was packed with  $\approx 1$  cm (*ca.* 0.8 mL) glass wool, followed by  $\approx 1.4$  g (*ca.* 1.2 mL) purified sea sand (analytically pure, acid-purified, calcinated). Approx. 105.3 mg of MWCNTs (*ca.* 2.8 mL; 1 sample bed volume) were added (Fig. 1). The column filling was lightly packed with a glass rod. Lastly, a second identical layer of sea sand was packed on top of the column. The total column packing volume was approx. 5–7 mL. Then, the column was filled with  $\approx 3$ –5 mL MilliQ water and allowed to equilibrate for 90–120 min. Subsequently, the column was flushed with  $\approx 14$  mL of water over approx. 30 min, which is equivalent to five times the column’s bed volume. The use of water volume significantly larger than the column’s internal space ensured a thorough washing of the column. A blank column, similarly packed but without the test material, was run in parallel. Consecutive samples ( $n = 5$ –7) of *ca.* 28 mL (corresponding to *ca.* 10 sample bed volumes) were collected from the



**Fig. 1** Column elution apparatus with column packing: 0.45  $\text{mL min}^{-1}$  [ $\approx 27 \text{ mL h}^{-1}$ ] was the high flow rate, 0.25  $\text{mL min}^{-1}$  [ $\approx 15 \text{ mL h}^{-1}$ ] the low flow rate. A 500 mL all-glass vessel was used as a reservoir for the MilliQ water, connected *via* glass joint to the column. SBV: sample bed volume.

continuous flow column. Two flow rates were investigated: 0.45  $\text{mL min}^{-1}$  (collected over *ca.* 1 hour) and 0.25  $\text{mL min}^{-1}$  (collected over 2 hours). Collected samples were directly analysed using a total organic carbon (TOC) analyser applying the NPOC method.

### Dispersion stability

The dispersion stability of MWCNTs was investigated in simulated environmental media, using the screening test outlined in OECD TG 318 (ref. 18) and OECD GD 318.<sup>4</sup> Results are primarily reported for K-Nanos, since Jenotubes were tested at an earlier stage when limited experience was available. This resulted in some unfavourable test conditions, especially for Jenotube 8 (tested as the first nanoform in this series). Nonetheless, results for Jenotubes are presented and briefly discussed in the respective section.

Depending on the K-Nanos MWCNT form, 52.6–93.22 mg were introduced into a 250 mL Erlenmeyer flask and pre-wetted using 1 mL of ultrapure water and 1 mL of ethanol (99.8%, Chemsolute) for 24 h at 25 °C in a covered climate chamber. The ethanol was added to enhance the wetting of the tubes, given their narrow inner volume. Stock solutions were prepared in a total volume of 125 mL ultrapure water yielding a 10 times concentrated stock solution (relative to the sample particle concentration used in the test). A calorimetrically calibrated probe sonicator (Bandelin



SONOPLUS 2200.2 series, with a VS 70 T sonicator probe, 13 mm diameter) was used, setting the amplitude to an instantaneous sonication power<sup>20</sup> of  $0.16 \text{ W mL}^{-1}$ , equivalent to the energy density  $[\text{W s mL}^{-1}]$ .<sup>21</sup> Sonication of stock suspensions was performed in an ice bath with the probe immersed to a depth of 2.5 cm for 600 s, delivering a total energy delivered to the suspension of  $96 \text{ J mL}^{-1}$ . The simulated environmental media were prepared in ultrapure water containing  $10 \text{ mg L}^{-1}$  dissolved organic carbon (DOC) from Suwannee River natural organic matter (NOM). Varying electrolyte ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ) concentrations of 0 mM, 1 mM, and 10 mM were used at pH 4, 7, and 9. The study involved nine test conditions, each prepared in triplicate, totalling 27 tubes of 50 mL. A particle number concentration of  $\approx 5 \times 10^{12}$  MWCNT particles per L was used (SI Table S3). The appropriate mass concentration to yield the target concentration of  $5 \times 10^{12}$  particles per L was calculated based on the MWCNT density and the calculated volume for a single tube, most probably overestimating the real particle concentration (see SI for details). Stock solutions were continuously stirred during application to each test tube to prevent early agglomeration of the test material (SI Fig. S2). Dispersion stability was measured at 0 and 6 hours. At the 0-hour mark, 0.25 mL samples were carefully taken from the top 0.5–1 cm of the liquid column from the test tube and transferred to 96-well plates. This deviation from TG 318 (0.5 mL sample volume) results from the maximum possible volume per well in the plate reader. We favoured this approach as it allowed for the rapid determination of dispersion stability prior to any sedimentation of the MWCNTs occurring. This adaptation was judged to be without detrimental effect on measured values. After 6 hours (SI Fig. S1 and S3), the tubes were centrifuged at 485 g for 10 minutes to ensure settling of larger agglomerates ( $>1 \mu\text{m}$ ). Then, 0.25 mL samples were carefully drawn from the top 2 cm of the supernatant. Collected samples were immediately analysed for MWCNT particle concentration using a UV-vis spectrophotometer plate reader (Tecan, SPARK), facilitating the almost simultaneous and immediate analysis of the 96-well plates. MWCNTs exhibit a small absorbance peak at 550 nm, which was utilised for quantification. A standard curve of mass concentration *versus* absorbance at 550 nm (with NOM at  $10 \text{ mg L}^{-1}$  DOC; see above) was used to convert absorbance values into particle number concentration and to calculate the percentage dispersion stability. Absorbance at 550 nm was not impacted by the presence of the electrolyte (details not shown).

### Degradation and adsorption/desorption screening

**Experimental set-up: continuous activated sludge test.** A pre-phase study was conducted following OECD TG 303A<sup>19</sup> using the continuous activated sludge test. The study utilised Jenotube 8 MWCNTs. Test suspensions were prepared (after pre-wetting) at 3, 30, and  $300 \text{ mg L}^{-1}$  by mixing Jenotube 8 MWCNTs in demineralised water with the addition of non-

ionic surfactant Tween 80 ( $300 \text{ mg L}^{-1}$ ). After being thoroughly stirred, the suspension was sonicated. This was performed using either an ultrasonic water bath (40 W) or a probe sonicator (40 W), with the probe tip placed 2.5 cm below the surface.

Secondary activated sludge was obtained from the Nieuwgraaf wastewater treatment plant (Duiven, The Netherlands), which primarily treats domestic wastewater. Effluent was obtained from a lab-scale sequencing batch reactor inoculated with secondary activated sludge and daily dosed with primary settled domestic wastewater. The effluent was considered comparable to the effluent described in OECD TG 303A. In the simulated sewage treatment plant (STP) system, the test material was dosed to the continuous activated sludge (CAS) reactor *via* a syringe pump (SI Fig. S4). A stirring bar inside the syringe ensured continuous mixing of the suspension during dosing. The feasibility of a spectroscopic analysis of MWCNTs in effluent was assessed by spiking MWCNT stock suspension into the activated sludge treatment plant effluent.

**Analytical methods and calibration.** Calibration curves for UV-vis spectrophotometric analysis were prepared from a  $50 \text{ mg L}^{-1}$  Jenotube 8 MWCNT stock suspension containing  $500 \text{ mg L}^{-1}$  Tween 80. The stock suspension was prepared by wetting MWCNTs in small volumes of demineralised water for 24 hours. The wetted MWCNTs were then diluted in a Tween 80 solution to the target concentration, mixed for 2 hours, and subsequently ultrasonicated in a water bath for 30 minutes, followed by additional 48 hours of stirring. Calibration curve standards were prepared from this stock suspension shortly after a second 30-minute ultrasonic treatment in the water bath and under continuous mixing on a stirrer plate. In total, two calibration curves were prepared from two separate stock suspensions (SI Fig. S5). MWCNT suspensions were analysed using a UV-vis spectrophotometer (HACH, DR6000) at a wavelength of 550 nm with 5 cm path length cuvettes.

**Microscopy.** Jenotube 8 MWCNT samples were examined by scanning electron microscopy (SEM) and optical microscopy both 'as-is' and after preparation as a slurry. The slurry was created by dispersing a spatula-tip amount of material in 5 mL of water *via* ultrasonic bath sonication (5 min). SEM images were captured on a Zeiss Crossbeam 540 FEG-SEM (Field Emission Gun, 1 kV, 250pA, WD 3 mm). The untreated sample was affixed to an aluminium stub with a carbon tab, and the droplet of slurried sample was dried on a separate aluminium SEM specimen stub. To prevent electron beam charging, samples were C-coated (12 nm). Optical images of slurried MWCNT samples were acquired using a Zeiss AxioImager (10 $\times$  and 50 $\times$  objectives). To assess the detectability of MWCNTs in activated sludge *via* Raman microscopy, untreated Jenotube 8 MWCNTs and a droplet of sludge were analysed using a Witec Alpha 300 RA microscope. Parameters included a 532 nm laser (1 mW



power, 0.5 s exposure, single spectrum (128 scans co-addition), 50×–100× magnification).

**Isotopic analysis.** Isotopic signatures of K-Nanos and Jenotube MWCNTs were experimentally determined to evaluate the feasibility of tracking the fate of MWCNTs within STP systems. Carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ , expressed as  $\delta^{13}\text{C}\text{-TC}$  (Total Carbon)) were analysed using Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS), referenced to the Vienna Pee Dee Belemnite (VPDB) standard, with an analytical precision of  $1\sigma \pm 0.3\%$ .

## Results

### Water solubility or dissolution of nanomaterial

The differences in carbon concentrations between samples and blanks for both flow experiments ( $0.45\text{ mL min}^{-1}$  and  $0.25\text{ mL min}^{-1}$ ) were not statistically significant for all tested nanoforms (*t*-test two-sample assuming unequal variances;  $p > 0.05$ ). Limit of detection (LOD) and limit of quantification (LOQ) for the newly developed method were established at  $50.7\text{ }\mu\text{g C L}^{-1}$  (LOD) and  $126.4\text{ }\mu\text{g C L}^{-1}$  (LOQ) for the K-Nanos MWCNTs experiment, and at  $57.4\text{ }\mu\text{g C L}^{-1}$  (LOD) and  $141.2\text{ }\mu\text{g C L}^{-1}$  (LOQ) for the Jenotubes MWCNTs experiment. For all K-Nanos and Jenotube forms, measured carbon concentrations (blank-corrected) were determined as  $<\text{LOD}$ , *i.e.*,  $<50.7\text{ }\mu\text{g C L}^{-1}$  and  $<57.4\text{ }\mu\text{g C L}^{-1}$ , respectively. Thus, no detectable dissolution of carbon from MWCNT test material compared to the blank was observed (Table 1).

### Dispersion stability

The dispersion stability of K-Nanos MWCNT nanoforms was consistently low, measured at  $<10\%$  (ranging from 0–8.7%) across all nine test conditions, involving varying electrolyte concentrations and pH values. This places K-Nanos MWCNTs in the ‘low dispersion stability’ category according to OECD TG 318. For Jenotube MWCNT nanoforms, dispersion stability was also  $<10\%$  (ranging from 0–6.3%), with the exception of Jenotube 8, which ranged from 14.2–17.2%. However, this higher value for Jenotube 8 was attributed to

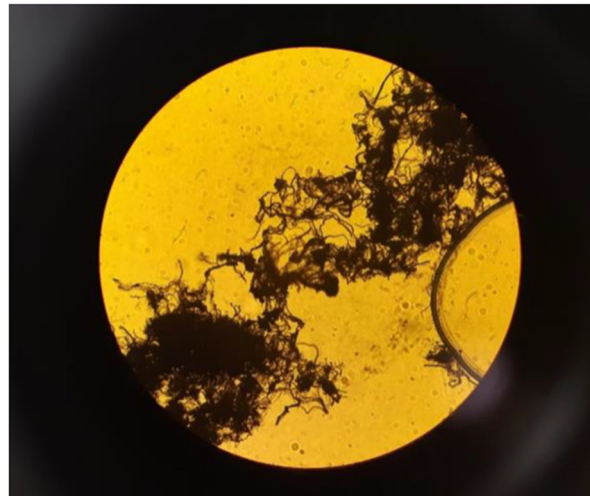


Fig. 2 Optical microscope image of agglomerated Jenotubes 8 MWCNTs sampled from  $300\text{ mg L}^{-1}$  stirred MWCNT suspension ( $20\times$  magnification).†

an excessively low test concentration, leading to an overestimation of stability (due to an overestimated density for Jenotube 8, see SI Table S3). Visual observations revealed that the here investigated MWCNT samples rapidly formed inhomogeneous suspensions characterised by loose, visible agglomeration, which settled at the bottom of the test vial (SI Fig. S1 and S3). Visual observation of the stock solution also showed that K-Nanos and Jenotube MWCNTs formed uniformly spread agglomerates very quickly (within the first few minutes), after which the process slowed (Fig. S3). In summary, no stable dispersion could be obtained, and both K-Nanos and Jenotube MWCNTs were considered non-dispersible.

### Degradation and adsorption/desorption screening

**Continuous activated sludge test.** When Jenotube 8 MWCNTs were dosed at 3, 30, and  $300\text{ mg L}^{-1}$  in demineralised water, large agglomerates formed quickly at all test concentrations. Even stirring the mixtures for  $\geq 72$  hours failed to disperse these agglomerates (Fig. 2). Agglomerates were also clearly formed at the lowest MWCNT concentration. Ultrasonic dispersion and the method described in the section on dispersion stability initially reduced the number of agglomerates; however, they reappeared rapidly, and no stable dispersion could be achieved. Also, continuous, prolonged ultrasonication failed to improve the dispersion stability. The addition of Tween 80 enhanced the homogeneity of the test suspensions and slowed the re-agglomeration after ultrasonication. Despite this, dosing MWCNTs at  $300\text{ mg L}^{-1}$  in a mixture with  $300\text{ mg L}^{-1}$  Tween 80 proved impractical. Although the dispersion remained stable in the syringe used for dosing, agglomerates reformed

Table 1 Mean carbon concentrations of MWCNT samples and blank measurements ( $n = 5\text{--}8$ )

Sample	Test item		Blank	
	Mean C conc. [ $\mu\text{g L}^{-1}$ ]		Mean C conc. [ $\mu\text{g L}^{-1}$ ]	
Flow rate [ $\text{mL min}^{-1}$ ]	0.45	0.25	0.45	0.25
K-Nanos 100	82.1	134.1 <sup>a</sup>	$107.2 \pm 52$	$107.5 \pm 89$
K-Nanos 210	55.6	68.7		
K-Nanos 300	63.2	106.9		
Jenotube 6	112.7	70.6	$108.6 \pm 50$	$86.2 \pm 31$
Jenotube 8	123.5	53.8		
Jenotube 10A	70.6	70.6		
Jenotube 10B	49.7	62.2		

K-Nanos: LOD:  $50.7\text{ }\mu\text{g C L}^{-1}$ ; LOQ:  $126.4\text{ }\mu\text{g C L}^{-1}$ . Jenotubes: LOD:  $57.4\text{ }\mu\text{g C L}^{-1}$ ; LOQ:  $141.2\text{ }\mu\text{g C L}^{-1}$ . Values  $<\text{LOD}$  were replaced by  $\text{LOD}/2$ ; values  $<\text{LOQ}$  were replaced by  $\text{LOQ}/2$ . Conc.: concentration. <sup>a</sup> Average conc.  $>\text{LOQ}$  ( $n = 5$ ), experimental artefact.

† Nouryon Specialty Chemicals B.V. (2022), commissioned by TSafeE GmbH on behalf of JEIO Co., Ltd.



quickly in the STP system's syringe tubing. Rapid agglomeration led to clogging, a problem aggravated by the low pumping speed of  $9.6 \text{ mL day}^{-1}$ , as demanded by the test guideline.

**UV-vis spectroscopy.** Linear calibration was necessary for the analysis of MWCNT concentrations in effluent samples. Linear calibration curves were successfully established for suspended Jenotube 8 MWCNTs in demineralised water, covering concentrations from  $10\text{--}100 \text{ mg L}^{-1}$ . The linearity of the curves suggested a homogeneous MWCNT stock suspension, implying that calibration standards could be reliably prepared *via* pipetting (SI Fig. S5). However, significant variations between the respective calibration curves derived from two different stock suspensions were observed (both prepared identically with  $50 \text{ mg L}^{-1}$  MWCNTs and  $500 \text{ mg L}^{-1}$  Tween 80). Despite their visual similarity, these differences challenge the reproducibility of the suspension preparation method. Analysis of MWCNTs in the  $10\text{--}100 \text{ mg L}^{-1}$  range proved unfeasible because the absorbance at  $550 \text{ nm}$  lacked sufficient specificity, overlapping with the effluent's inherent absorbance.

**SEM analysis.** SEM images of dry Jenotube 8 MWCNTs showed agglomerates of MWCNT bundles (Fig. 3). These agglomerates were visible to the naked eye and up to hundreds of micrometres in length (Fig. 3a and b). At higher magnification, images showed that MWCNT bundles consisted of thin, individual MWCNTs (Fig. 3c and d). SEM images of the dried aqueous suspension (prepared in demineralised water using ultrasonic dispersion) revealed a thin layer of larger agglomerates, with hardly any individual nanotubes visible. While ultrasonic dispersion reduced the size of MWCNT agglomerates, it most likely did not destroy the nanotubes. However, it was challenging to definitively assess the full impact of ultrasonic dispersion on the MWCNTs. This is because most of the material in the

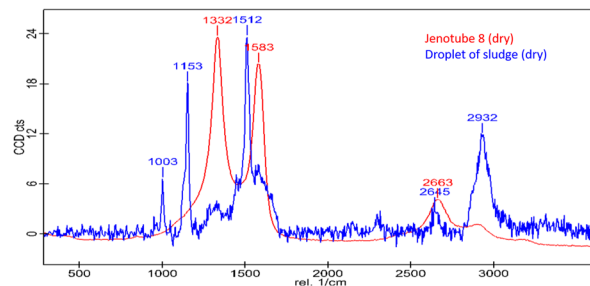


Fig. 4 Raman spectra of Jenotube 8 MWCNTs (red) and dried sludge (blue).†

samples remained as small agglomerates, and no untreated, untangled MWCNT material was available for comparison. Optical microscopy of the aqueous MWCNT suspension confirmed the presence of agglomerates even in the slurried state (see Fig. 2 after stirring), indicating they did not reform only during drying.

**Raman spectroscopy.** The Raman spectra for Jenotube 8 MWCNTs and activated sludge are depicted in Fig. 4, showing clearly distinct signals. As for the Raman shift of MWCNTs, the G-band ( $\sim 1580 \text{ cm}^{-1}$ ) represents the  $\text{sp}^2$  hybridised carbon atoms within the highly ordered, crystalline lattice of the carbon nanotubes (CNTs), while the D-band ( $\sim 1330 \text{ cm}^{-1}$ ) is associated with structural defects or disordered carbon, such as amorphous structures.<sup>22,23</sup> This characteristic Raman shift was measured at  $1583 \text{ cm}^{-1}$  (G-band) and  $1332 \text{ cm}^{-1}$  (D-band) for Jenotube 8 MWCNTs (Fig. 4). The G-band points towards the highly ordered, multi-layered crystalline structure of the sample (% crystallinity reported in SI Table S2). However, the activated sludge contained components that would cause disturbances in the Raman signal at the characteristic MWCNT band positions (Fig. 4). This spectral overlap, together with an inconsistent background signal of the activated sludge, precluded any quantitative analysis. Even a small amount of sludge particles in the effluent could disrupt the signal through phenomena such as fluorescence. Nevertheless, qualitative detection of MWCNTs remained possible by correlating structural differences observed in the optical image with their corresponding Raman spectra,<sup>22</sup> as demonstrated in Fig. 5. Specifically, the yellow and blue spots were identified as Jenotube 8 MWCNTs test material.

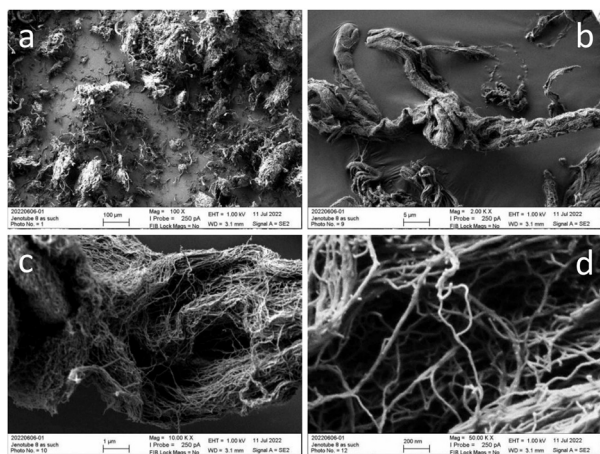


Fig. 3 SEM images of Jenotube 8 MWCNTs 'as-is' (untreated, dry) at different magnifications showing: a) and b) macroscopically visible agglomerates of MWCNT bundles, hundreds of micrometres in size; c) and d) individual tubes within MWCNT bundles at higher magnification ( $\times 1000$  and  $\times 50\,000$  magnification, respectively).†

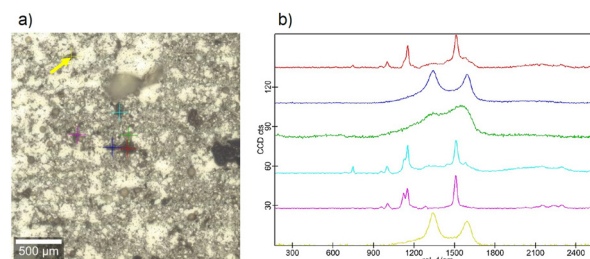


Fig. 5 Optical image from a Raman microscope: a) selected data points for Raman spectral analysis; b) Raman spectra with colours corresponding to the selected points in the left image a).†



Consequently, while Raman microscopy is a viable tool for the qualitative detection of MWCNTs in activated sludge, quantitative analysis appeared unfeasible under these test conditions.

**Stable isotopes of carbon  $^{13}\text{C}/^{12}\text{C}$ .** The  $\delta^{13}\text{C}$  values of K-Nanos and Jenotube MWCNTs were as follows: K-Nanos 100:  $-29.0\text{‰}$ ; K-Nanos 210:  $-29.2\text{‰}$ ; K-Nanos 300:  $-29.4\text{‰}$ ; Jenotube 6:  $-29.5\text{‰}$ ; Jenotube 8:  $-30.6\text{‰}$ ; Jenotube 10A:  $-27.4\text{‰}$ ; and Jenotube 10B:  $-27.9\text{‰}$ .

## Discussion

### Water solubility and dissolution rate according to REACH annex VII, section 7.7

Carbon is ubiquitously found in the environment and may be released from laboratory equipment, which makes it challenging to measure carbon dissolved from carbon-based nanomaterials in environmental media. Despite existing knowledge that MWCNTs are insoluble, providing data on water solubility or nanomaterial dissolution is a mandatory requirement under the REACH regulation. For substances that are 'insoluble' in water, a limit test up to the detection limit of the analytical method is required, as specified in REACH annex VII, column 2. Consequently, we developed a novel methodology integrating OECD TG 105 (ref. 16) and OECD GD 318 (ref. 4), using either carbon-free materials or those for which the release of carbon-containing compounds can be largely ruled out. We based this approach on findings from preliminary studies. For example, nano-sized filters (5 kDa MW cut-off; polyethersulfon) used to separate non-dissolved MWCNTs from water were found to release a relevant degree of carbon (*ca.*  $200\text{ }\mu\text{g L}^{-1}$ ), even after the membranes had been stored for three weeks in ultrapure water and had been subsequently washed by 15 centrifugation cycles using fresh ultrapure water. In the case of MWCNTs, the elimination of a nano-filter from the experimental set-up was possible. The high MWCNT length and their aggregated/agglomerated state prevented passage through standard glass frits (pore width 160–250  $\mu\text{m}$ ), supported by the barrier of glass wool and sea sand. We were able to eliminate carbon contamination from laboratory equipment effectively, as evidenced by the measured carbon concentrations in Table 1; all values were below the background carbon levels of the ultrapure water ( $<200\text{ }\mu\text{g L}^{-1}$ ) used throughout the study. This new method is characterised by a low limit of detection for carbon. It allowed demonstrating the (carbon-)insolubility for all tested MWCNTs. Since the measured concentrations from the test materials were not significantly different from the blanks, measured carbon concentrations are attributed to the background present in ultrapure water.

Furthermore, very sensitive NPOC analysis was applied with LOD and LOQ values of about  $50\text{--}55\text{ }\mu\text{g C L}^{-1}$  and  $125\text{--}140\text{ }\mu\text{g C L}^{-1}$ , respectively. These values are significantly lower than LOD and LOQ values reported in the scientific literature, such as  $100\text{ }\mu\text{g C L}^{-1}$  and  $800\text{ }\mu\text{g C L}^{-1}$ ;  $96\text{ }\mu\text{g C L}^{-1}$  and  $480\text{ }\mu\text{g C L}^{-1}$ ;  $25$  and  $160\text{ }\mu\text{g C L}^{-1}$

and  $800\text{ }\mu\text{g C L}^{-1}$ ,<sup>26,27</sup> respectively. Thus, we present a novel method for the determination of carbon dissolution into water applicable to certain carbon-based nanomaterials with similar physicochemical properties.

### Partition coefficient *n*-octanol/water or dispersion stability according to REACH annex VII, section 7.8

As MWCNTs are considered as inorganic nanomaterials, the partition coefficient *n*-octanol/water ( $\log K_{ow}$ ) is not applicable. Instead, dispersion stability becomes the relevant physicochemical parameter of interest. A primary challenge in measuring MWCNT dispersion stability lies in the analytical method to detect the test material in the media. The inherently low MWCNT concentrations, and the difficulty in preparing a stable dispersion, made TOC analysis impracticable. This was due to the rapid settling of the sample material during analytical measurement, making it inaccessible to both the autosampler and the analytical device. Furthermore, the presence of natural organic matter (NOM) in the test medium interfered with the measurements and led to high background values. Therefore, test material concentrations were analysed using UV-vis spectrophotometry. Measurements were performed with a spectrophotometer plate reader, which facilitated the almost simultaneous and immediate analysis of 27 samples with 9 replicate measurements, directly after 0- or 6-hours incubation times. This method appears currently unique in its ability to enable immediate analytical determination of multiple samples following incubation, effectively overcoming the challenges of rapid settling and agglomeration of the test material. We deemed this method appropriate and sufficiently sensitive to measure 10% of the initial material concentration. For example, for the K-Nanos MWCNT concentration of  $43.79\text{ mg L}^{-1}$  (corresponding to the targeted  $5 \times 10^{12}$  particles per L), the achieved LOD and LOQ were  $0.54\text{ mg L}^{-1}$  and  $2.01\text{ mg L}^{-1}$ , respectively. Our methodology involved significant practical adaptations to the analytical approach of OECD TG 318 to accommodate the unique challenges presented by MWCNTs, particularly their propensity for rapid settling and agglomeration.

Another challenge in MWCNT investigation is the preparation of stock dispersions. OECD TG 318 provides recommendations for preparing stock dispersions by probe sonication, emphasising the need for cautious sonication procedures for high-aspect-ratio nanomaterials to avoid their destruction (breakage). Indeed, as determined by Fuge *et al.*,<sup>28</sup> applying an energy input of only 1.2 kJ ( $68.6\text{ J mL}^{-1}$ ) to a 17.5 mL aqueous dispersion of 0.005% (w/w) undoped MWCNT already dramatically decreased the arithmetic mean tube length, as determined by SEM (from close to 1000  $\mu\text{m}$  to 17.1  $\mu\text{m}$ ). Increasing the energy input to 6 kJ ( $342.9\text{ J mL}^{-1}$ ), by prolonging the sonication time to 180 s, resulted in a further reduction of the mean length to 12.2  $\mu\text{m}$ . Considering that the original tube length of K-Nanos is *ca.* 50  $\mu\text{m}$ , a total energy delivered to the suspension of  $96\text{ J mL}^{-1}$  was judged



to be an acceptable compromise to yield sufficient dispersion while minimising destruction/breakage of the tubes. Because OECD TG 318 recommends a longer dispersion time of 600 s than applied by Fuge *et al.*,<sup>28</sup> and the time integrated total energy ( $\text{J mL}^{-1}$ ) is decisive for the dispersion result, we reduced the instantaneous sonication power to  $0.16 \text{ W mL}^{-1}$ . This yielded the target total energy of  $96 \text{ J mL}^{-1}$  (which falls between the conditions mentioned by Fuge *et al.*<sup>28</sup>). The associated decreased instantaneous sonication power ( $0.16 \text{ W mL}^{-1}$  compared to *ca.*  $1.9 \text{ W mL}^{-1}$  according to Fuge *et al.*<sup>28</sup>) may also have resulted in a decreased risk of tube breakage. Our investigations confirm that using adequate measures for the sonication energy delivered to the suspension (*i.e.*, instantaneous sonication power ( $\text{W mL}^{-1}$ ) and time-integrated total energy delivered to the suspension ( $\text{J mL}^{-1}$ )), as well as using a comparable volume (125 mL recommended by OECD TG 318) is highly important for reproducible results.

Another important aspect is the particle concentration. To convert the mass concentration of the stock suspension to a particle concentration, reliable data on density and particle size are decisive. As outlined in more detail in the SI, dynamic light scattering (DLS)—the method suggested by OECD TG 318 for determining the particle size in stock dispersions—cannot be performed for MWCNTs. Deriving particle concentrations using the calculated single MWCNT volume instead—as done in this study—is a viable solution to this problem. However, it may significantly overestimate the actual particle concentration because assuming sonication to result in single MWCNT in dispersion is not realistic. Due to the inverse relationship between particle concentration and dispersion stability, overestimation of particle concentration is equivalent to overestimating dispersion stability. Overall, these adaptations allowed us to generate meaningful and conservative data on the dispersion stability of investigated MWCNTs, even when standard quantitative measures proved unfeasible.

**Experimental findings and implications for further testing.** The experimental results consistently demonstrated that both K-Nanos and Jenotube MWCNTs are non-dispersible. This finding was consistently confirmed by optical microscopy observations during both the dispersion stability and STP feasibility studies. The inability to prepare a stable dispersion highlights a major obstacle in testing these materials. Without a stable dispersion or suspension, the applicability of standard guidelines is severely limited, unless specific guidance documents or test protocols are available regarding the preparation of nanomaterial dispersions or methods for working with unstable test suspensions during testing. The recently published OECD Guidance Document (GD) No. 420 (ref. 29) on sediment and aquatic toxicity testing of nanomaterials provides guidance on how to prepare and work with nanomaterial dispersions in relevant OECD TGs (*e.g.*, OECD TG 201, TG 202, or TG 203). The same holds true for *in vitro/in chemico* human health assays, such as those for genotoxicity or skin sensitisation; if no

nanomaterial-specific dispersion protocols are available, most assays requiring a stable dispersion are inapplicable. (The assessment of the applicability of standardised *in vitro/in chemico* human health assays to MWCNTs is the subject of the companion article<sup>15</sup>).

### Degradation and adsorption/desorption screening according to REACH annex VIII, sections 9.2 and 9.3

**Feasibility study of an STP simulation test for investigating the environmental fate of MWCNTs.** The feasibility of conducting an STP simulation study depends on the availability of a suitable analytical method for the test material. We investigated various analytical approaches (practical and theoretical) to establish the feasibility of measuring MWCNTs in an STP simulation study, following the OECD TG 303A ‘Simulation Test—Aerobic Sewage Treatment—Activated Sludge Units’.<sup>19</sup> Moreover, we developed a simplified theoretical mass balance model for OECD TG 303A, which simulates the potential fate of MWCNTs based on given experimental data and theoretical model assumptions (SI Fig. S6 and S7).

**Analytical challenges and alternatives.** Distinguishing and quantifying MWCNTs against the high carbon background proved challenging because carbon is omnipresent in sewage, sludge solids, and effluent. Given their insolubility, poor water dispersibility, and high density, MWCNTs were considered to primarily distribute with the activated sludge solids fraction within STP systems, similar to what has been demonstrated for  $\text{TiO}_2$  nanoparticles.<sup>30</sup> Moreover, given their hydrophobic nature, we expected MWCNTs to show considerably more pronounced adsorption to the organic carbon fraction of activated sludge solids compared to metal and metal oxide nanoparticles.<sup>31,32</sup> The standard method prescribed by OECD TG 303A for assessing test concentrations is the measurement of dissolved and total organic carbon. However, since MWCNTs are a carbonaceous substance, the total organic carbon (TOC) adsorbed to activated sludge cannot be distinguished from the TOC of the activated sludge itself. Although relatively stable and homogeneous MWCNT suspensions could be prepared with Tween 80 for the experimental work of the feasibility study, dosing these suspensions into the CAS unit proved impossible due to rapid formation of MWCNT agglomerates within the syringe pump tubing. Manual dosing of the MWCNT suspension to the CAS reactor could be another option; however, the reproducibility of a manually dosed MWCNT content would be questionable due to differences already observed between freshly prepared MWCNT stock suspensions and their agglomeration over time.

The analytical alternatives to TOC analysis for the detection of MWCNTs assessed here included UV-vis spectroscopy, SEM microscopy, and Raman spectroscopy. Unfortunately, these three techniques proved insufficiently sensitive to discriminate the test material from the high carbon background of the test media when investigated with



MWCNT suspension-spiked effluent samples. Moreover, impurities in the test matrix complicated the identification and quantification of MWCNTs. The microscopic techniques inherently offered only qualitative analysis of MWCNTs. Both optical microscopy and SEM lack specificity, and constituents within the effluent matrix can significantly hinder MWCNT identification.<sup>8</sup> Given the expected low MWCNT concentrations in the CAS test effluent, a concentration step would be necessary for qualitative assessment using these methods. Also concentrating the effluent would enrich existing solids and impurities, further complicating microscopic analysis.

While Raman is highly specific for the sp<sup>2</sup> carbon lattice of MWCNTs, its sensitivity is severely limited in high carbon backgrounds such as activated sludge. Biogenic matrices, including sewage and sludge solids, contain numerous fluorophores that emit broad, intense signals when excited by Raman lasers, often overwhelming the weaker Raman scattering.<sup>33,34</sup> Activated sludge is particularly rich in organic matter such as extracellular polymeric and humic substances. The latter are noted for their ability to adsorb onto carbon CNT surfaces, which can lead to quenching in the measured spectral response.<sup>35</sup> Literature suggests that these biogenic components exhibit broad fluorescence that can mask the characteristic graphitic peaks of CNTs, complicating quantitative detection.<sup>36</sup> The high background from biogenic sources reduces the signal-to-noise ratio (SNR), making it difficult to detect CNTs at environmentally relevant (low) concentrations.<sup>8,33</sup> This is consistent with our findings: while structural differences in optical images allowed for qualitative correlation, the inconsistent background signal from the sludge particles precluded a reliable quantitative method.

We concluded that environmental fate testing of MWCNTs using UV-vis spectroscopy, SEM analysis, and Raman spectroscopy was not feasible in an STP simulation study according to OECD TG 303A, even if appropriate dosing had been feasible. Isotope ratios ( $\delta^{13}\text{C}$ ) represent a viable alternative, as they provide a mass-balance approach that optical techniques like Raman cannot offer in the context of non-homogeneous, biogenic slurries.

**Use of stable isotope ratios and metal impurities as tracers.** Isotopic signatures were investigated as a potential analytical means to monitor the environmental fate of MWCNTs in an STP simulation.<sup>8</sup> The method relies on the premise that the differences between the stable carbon isotope ratios (<sup>12</sup>C and <sup>13</sup>C) of MWCNTs and sewage sludge could allow the tracking of MWCNT accumulation. We investigated the applicability of this method through experimental determination of isotopic ratios for the tested MWCNT nanoforms and theoretical considerations of MWCNT-enriched effluent over time, depending on sample dosing and sludge feeding to the STP system (SI Fig. S6 and S7). CNTs may be significantly depleted in their  $\delta^{13}\text{C}$  content because their production is based on largely fossil feedstock, in contrast to environmental biogenic matrices. The feasibility of basing the analytical determination of MWCNTs on isotopic signatures thus depends on a significant difference in  $\delta^{13}\text{C}$  values between MWCNTs and sewage sludge. The isotopic signature  $\delta^{13}\text{C}$  is determined by the ratio *R* of the two stable carbon isotopes <sup>13</sup>C/<sup>12</sup>C and is reported in permille (‰) relative to a VPDB reference material (eqn (1)).

$$\delta^{13}\text{C} = \left( \frac{R_{\text{sample}}}{R_{\text{VPDB}}} - 1 \right) \times 1000; R = {}^{13}\text{C}/{}^{12}\text{C} \quad (1)$$

Experimental determination showed that  $\delta^{13}\text{C}$  values for K-Nanos and Jenotubes ranged from -30.6 ‰ to -27.4 ‰. This range is similar to the  $\delta^{13}\text{C}$  values reported for activated sewage sludge, which typically range from -24.0 ‰ to -28.0 ‰ (Table 2, SI Table S4).<sup>37</sup> Based on available experimental  $\delta^{13}\text{C}$  values for activated sludge and MWCNTs, we made the simplified assumption of  $\delta^{13}\text{C}_{\text{MWCNTs}} = -30$  ‰ and  $\delta^{13}\text{C}_{\text{sludge}} = -25$  ‰, leading to a difference in isotopic signatures ( $\Delta\delta^{13}\text{C}$ ) of 5 ‰.

In a theoretical mass-balance approach for the STP simulation study, we assumed 99% adsorption and continuous dosing over time. We calculated the least significant difference (LSD) by applying the two-sample *t*-test principle to the combined uncertainties. This LSD defined the minimum  $\delta^{13}\text{C}$  shift necessary to statistically differentiate the baseline sludge (-25 ‰) from the MWCNT-enriched sludge. With the given model assumptions, the activated

**Table 2**  $\delta^{13}\text{C}$  values for STP sludges and K-Nanos and Jenotube MWCNTs

Samples	$\delta^{13}\text{C}$ [‰]	Reference
$\delta^{13}\text{C}$ values for STP sludge		
Activated sludge from 20 STPs - origin Germany	-24.0 to -28.0	UBA <sup>37</sup>
$\delta^{13}\text{C}$ values for MWCNTs, measured <sup>a</sup>		
K-Nanos 100	-29.2	Present study
K-Nanos 210	-29.0	
K-Nanos 300	-29.4	
Jenotube 6	-29.5	
Jenotube 8	-30.6	
Jenotube 10A	-27.4	
Jenotube 10B	-27.9	

<sup>a</sup> Standard deviation/standard error of measurement (SEM), method precision:  $1\sigma = \pm 0.3$  ‰.



sludge from the CAS reactor should consist of *ca.* 48% MWCNTs to ensure the isotopic shift is statistically significant (SI Fig. S7).

Thus, applying stable isotope analysis to carbon-based nanomaterials may be a feasible analytical method to determine the distribution of MWCNTs in an STP simulation study only if (1) high accumulation levels can be achieved, or (2) if there are greater differences between the isotopic signatures of MWCNTs (or SWCNTs) and activated sludge. Significantly more negative  $\delta^{13}\text{C}$  values for MWCNTs and SWCNTs have been reported elsewhere<sup>38</sup> (SI Table S4). Such variations depend on the carbon source used during the nanomaterial's production. Moreover, further refinement and evaluation of the model and its assumptions are necessary; specifically, verifying the assumed 99% adsorption rate and the plausibility of achieving high MWCNT fraction in the activated sludge required to produce a statistically detectable  $\delta^{13}\text{C}$  shift.

Isotopic signatures of  $\delta^{14}\text{C}$  are another option and allow to distinguish between biogenic and fossil carbon. This would necessitate determining the  $^{14}\text{C}/^{12}\text{C}$  ratio by accelerator mass spectrometry (AMS) after combustion to  $\text{CO}_2$ . Applying this method, the biogenic carbon content of activated sludge was determined to be approx. 80%.<sup>37</sup> In contrast, CNTs produced from fossil carbon feedstock should not contain  $^{14}\text{C}$ .

Another added complexity of using isotopic signatures was the anticipated change in the stable isotope ratios of the baseline sludge over the three-week test duration, caused by feeding the activated sludge with synthetic carbon sources (peptone, meat extract, and urea). Consequently, establishing a comprehensive carbon mass balance encompassing all these varied sources—activated sludge, surplus sludge, and MWCNTs—was deemed complex, leading to an unacceptable level of uncertainty for reliable application in a regulatory context.

Finally, while  $\delta^{13}\text{C}$  or  $\delta^{14}\text{C}$  isotopic analysis do not appear suitable as realistic monitoring tools to distinguish K-Nanos or Jenotubes from sewage sludge or effluent, isotopic signatures may be used as a method to investigate the MWCNT adsorption/accumulation properties by means of a statistically significant shift of  $\delta^{13}\text{C}$  that can be assessed by the mass balance approach. This represents a step towards a potential way to investigate the environmental fate of carbon-based nanomaterials. Moreover, because  $\delta^{13}\text{C}$  values for MWCNTs depend on their production's carbon source, the theoretical mass balance approach (SI Fig. S7) demonstrated the feasibility of using carbon stable isotope ratios to quantify carbon-based materials against the STP background, provided the nanomaterial's  $\delta^{13}\text{C}$  value differs significantly from the STP carbon sources. This important finding may allow the method's application for other carbon-based nanomaterials.

Lastly, the application of metal elements to track MWCNTs within the STP simulation system was considered, utilising the known metal impurities of MWCNTs as a tracer.

However, this method proved infeasible because metal impurities dissolve inconsistently from the CNT matrix, as indicated by available metal dissolution studies (unpublished). Thus, metal elements originating from MWCNT impurities cannot serve as a reliable quantitative method for mass-balancing MWCNT concentrations across the various test compartments/media.

#### Concluding on the feasibility of OECD TG 303 a study.

None of the available and investigated analytical methods proved easily applicable for measuring the assessed MWCNTs in an OECD TG 303A study. Consequently, this guideline study was considered technically not feasible to perform under the experimental set-up of this study. While applying a mass balance model and isotopic signatures remains a promising approach to investigate the environmental fate of MWCNTs in an STP simulation, an in-depth investigation of plausible model assumptions is necessary (*e.g.*, adsorption rate). For reasons discussed in the introduction, it was impossible to test additional experimental conditions, also considering that adequate analytical techniques are not available. Overall, it is currently impossible to conduct comprehensive environmental fate studies due to the lack of a quantitative analytical method capable of reliably analysing and monitoring the here-assessed MWCNTs in environmental media. This finding is in line with the ECHA-sponsored report noting that the '*current state of analytical methods reveals significant gaps in their ability to meet regulatory demands*'<sup>11</sup> for carbon-based nanomaterials. Beyond the ubiquitous carbon background, other components within complex environmental matrices—such as varying types of dissolved organic matter, inorganic salts, or suspended particulate matter—contribute to the complexity of analytical determination. Interference from such components can lead to false positives, quenching, or masking of MWCNT-specific signals (*e.g.*, UV-vis or Raman spectroscopy), thereby adding another layer of complexity to the accurate detection and quantification of MWCNTs in environmentally relevant samples. Standardised quantification methods must therefore be developed as a pre-requisite for any further refinement of test guidelines for MWCNTs.

## Conclusions

We provided a comprehensive, state-of-the-art evaluation of the applicability, feasibility, and suitability of standard test guidelines and analytical methods for meeting regulatory requirements for carbon-based nanomaterials, derived from practical, material-specific experience. Our work: (i) examined various approaches to overcome analytical challenges associated with the carbon-based nature of MWCNTs, (ii) acknowledged both the progress and limitations of the applied analytical methods, (iii) outlined potential novel approaches for future investigation, and (iv) provided the broader scientific community with



insights from the practical, real-world REACH registration of a carbon-based nanomaterial.

The current technical infeasibility of reliably quantifying MWCNTs in complex environmental media, as demonstrated in this study, poses a fundamental challenge to conducting robust environmental risk assessments under regulations such as REACH. Without reliable fate data, exposure modelling and subsequent risk characterisation become highly uncertain. This necessitates a discussion among regulatory bodies and industry stakeholders regarding alternative assessment strategies beyond standard methods, such as reliance on realistic exposure scenarios, read-across from analogous materials with better analytical methods, or the development of novel non-analytical assessment approaches.

Future research efforts should focus on highly sensitive, selective, and quantitative analytical techniques that can overcome the high carbon background and matrix interferences. This could include exploring advanced mass spectrometry techniques with specific labelling strategies for MWCNTs or developing novel optical probes that bind selectively to MWCNTs in complex matrices. Furthermore, research into tailored dosing systems that can reliably deliver highly agglomerating nanomaterials into dynamic environmental simulation systems remains critical. Finally, we consider a refinement and further development of the proposed mass balance model, coupled with the application of isotopic signatures, a promising environmental fate assessment tool. Overall, this research supports compliance professionals, regulatory bodies, and researchers in closing information gaps related to the detection and quantification of carbon-based nanomaterials for environmental fate assessment.

## Author contributions

Marie-Léonie Bohlen was responsible for conceptualisation, writing—original draft preparation, formal analysis, validation, visualisation, and supervision. She significantly contributed to writing—review & editing, and the methodology, focusing specifically on the formal analysis aspects of the OECD TG 303A adaptation and Raman spectroscopy. Markus Schwarz contributed significantly to conceptualisation, methodology, and validation. His key efforts focused on dispersion stability, nanomaterial dissolution, the feasibility study of the STP simulation, and the OECD TG 303A adaptation using isotopic signatures. He also contributed to writing—review & editing. Stefan Gartiser played a major part in the conceptualisation and methodology for the OECD TG 303A adaptation (study design) and assisted with writing—review & editing. Jan Oltmanns contributed to the methodology (dispersion stability, nanomaterial dissolution, and OECD TG 303A adaptation), and writing—review & editing. Hana Jo and Yeojin Lee contributed to writing—review, and project administration. Hyun Pyo Jeon contributed to funding acquisition.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5en01018g>.

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