Supporting Information for

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

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1 TEST MATERIAL CHARACTERISATION

Table S 1: K-Nanos MWCNTs summary of substance characteristics

Parameter	K- Nanos 100	K- Nanos 210	K- Nanos 300
Purity [% (w/w)]	94.00~97.00	91.95~98.00	95.00~98.60
Impurity, elements	Al, Co, Fe, Mg, Mo, V		
Single tube length [μm]	Mean: 46.22 Min.: 16.61 Max.: 89.89 D10: 25.44 D50: 46.59 D90: 78.43	Mean: 51.59 Min.: 25.22 Max.: 137.44 D10: 27.34 D50: 45.91 D90: 96.48	Mean: 62.76 Min.: 30.31 Max.: 134.81 D10: 37.50 D50: 55.42 D90: 119.68
Single tube diameter [nm]	Mean: 12.2 Min.: 7.68 Max.: 17.46 D10: 8.93 D50: 11.87 D90: 15.79	Mean: 12.13 Min.: 7.28 Max.: 19.68 D10: 8.05 D50: 12.08 D90: 16.30	Mean: 14.58 Min.: 7.36 Max.: 24.12 D10: 9.63 D50: 14.69 D90: 19.69
Aspect ratio [-] *(single tube length / single tube diameter)*	Mean: 3789 Min.: 2162 Max.: 5149	Mean: 4253 Min.: 3465 Max.: 6983	Mean: 4304 Min.: 4118 Max.: 5589
Bundle length [μm]	Mean: 41.997 Min.: 15.972 Max.: 70.859 D10: 24.47 D50: 43.30 D90: 61.82	Mean: 48.068 Min.: 26.239 Max.: 113.09 D10: 28.442 D50: 43.401 D90: 79.389	Mean: 54.852 Min.: 28.332 Max.: 104.916 D10: 35.055 D50: 49.980 D90: 93.144
Bundle diameter [μm]	Mean: 3.211 Min.: 0.445 Max.: 12.802 D10: 0.99 D50: 2.45 D90: 6.39	Mean: 2.968 Min.: 0.886 Max.: 15.441 D10: 1.168 D50: 2.207 D90: 5.327	Mean: 2.460 Min.: 0.70 Max.: 8.297 D10: 1.156 D50: 1.774 D90: 5.238
Number of walls	6−24	5−17	5−25
Crystallinity [%] (via Raman spectra)	97.00~98.40	97.80~98.60	97.00~98.20
Surface area [m²/g]	Mean: 200.8 Min.: 152 Max.: 287	Mean: 247.7 Min.: 202 Max.: 298	Mean: 228.3 Min.: 152 Max.: 348
Zeta potential [mV] *(in Ethanol)*	−0.459	−0.122	−0.176
Surface chemistry	no	no	no
Relative density [unitless]	1.761 ±0.030	-	-
Bulk density [g/cm³]	0.009 - 0.030	0.007 - 0.030	0.005 - 0.030

Note: D10, D50, D90: 10th, 50th, and 90th percentile of the particle size distribution.

Table S 2: Jenotube MWCNTs summary of substance characteristics

Parameter	Jenotube 6	Jenotube 8	Jenotube 10A	Jenotube 10B
Purity [% (w/w)]	98~99.2	98.5~99.7	97.8~99.2	97.3~98.9
Impurity, elements	Al, Co, Fe, Mg			
Single tube length [μm]	Mean: 77.62 Min.: 11.99 Max.: 238.86 D10: 30.10 D50: 68.34 D90: 151.17	Mean: 136.37 Min.: 2.00 Max.: 579.94 D10: 56.73 D50: 86.36 D90: 343.44	Mean: 35.87 Min.: 9.47 Max.: 107.21 D10: 15.01 D50: 28.60 D90: 66.40	Mean: 50.75 Min.: 10.92 Max.: 122.41 D10: 25.48 D50: 44.20 D90: 92.98
Single tube diameter [nm]	Mean: 7.4 ± 2.5 Min.: 4.4 Max.: 12.8 D10: 4.72 D50: 6.90 D90: 11.5	Mean: 8.04 ± 2.1 Min.: 5.1 Max.: 11.6 D10: 5.24 D50: 7.8 D90: 11.6	Mean: 11.0 ± 2.5 Min. 7.9 Max.: 14.2 D10: 7.9 D50: 11.6 D90: 14.1	Mean: 12.2 ± 3.3 Min.: 8.3 Max.: 17.9 D10: 8.8 D50: 11.9 D90: 15.9
Aspect ratio [-] *(single tube length / single tube diameter)*	Mean: 10490 Min.: 2726 Max.: 18661	Mean: 16961 Min.: 391 Max.: 49995	Mean: 3261 Min.: 1199 Max.: 7550	Mean: 4160 Min.: 1316 Max.: 6838
Bundle length [μm]	Mean: 68.6 ± 48.6 Min. 10.6 Max.: 211.1 D10: 26.6 D50 60.4 D90: 133.6	Mean: 129.8 ± 123.0 Min.: 1.9 Max.: 552.0 D10: 54.0 D50 82.2 D90: 326.9	Mean: 35.6 ± 21.0 Min.: 9.4 Max.: 106.4 D10: 14.85 D50 28.39 D90: 65.87	Mean: 48.8 ± 22.2 Min.: 10.5 Max.: 117.7 D10: 24.5 D50 42.5 D90: 89.4
Bundle diameter [μm]	Mean: 2.9 ± 1.7 Min.: 0.6 Max.: 8.8 D10: 1.58 D50 2.48 D90: 5.31	Mean: 3.0 ± 2.3 Min.: 0.33 Max. 7.93 D10: 0.39 D50 2.53 D90: 5.59	Mean: 6.3 ± 4.4 Min.: 1.1 Max.: 15.24 D10: 1.4 D50 4.5 D90: 14.5	Mean: 5.7 ± 3.2 Min.: 1.1 Max.: 15.4 D10: 2.6 D50: 5.2 D90: 10.1
Number of walls	3−9	5−8	5−12	7−18
Crystallinity [%] (via Raman spectra)	95.80	96.99	96.99	97.80
Surface area [m²/g]	Mean: 644 Min.: 576 Max.: 742	Mean: 570 Min.: 541 Max.: 618	Mean: 257 Min.: 223 Max.: 278	Mean: 217 Min.: 181 Max.: 249
Zeta potential [mV] *(with PVP in aqueous solution)*	−58.2	−20.9	−53.5	−28.4
Surface chemistry	no	no	no	no
Relative density [unitless]	-	2.294 (2.174−2.414)	-	-
Bulk density [g/cm³]	ca. 0.025	ca. 0.008	ca. 0.013	ca. 0.065

Note: D10, D50, D90 −10th, 50th, and 90th percentile of the particle size distribution.

2   CARBON DISSOLUTION

The following column with glass frit (porosity 0), (Fa. Lenz Laborglas, Germany: lenz-laborglas.de) was used: 20 cm length × 10 mm diameter, 15 mL volume, with glass frit and PTFE stopcock. Porosity 0 corresponds with P 250 (according to ISO 4793), equivalent to a pore width of 160-250 μm.

3   DISPERSION STABILITY

According to OECD TG 318, Dynamic Light Scattering (DLS) should be used to determine the particle size in the stock dispersion (ISO 22412:2008). However, DLS is inappropriate for very high aspect ratio nanomaterials like the MWCNTs investigated here.

Because of this, for the calculation of the appropriate mass concentration to obtain a particle target concentration of 5.00E12 particles/L, the volume for a single MWCNT was calculated from the dimensions of a single tube (note: the single tube length was determined only after finalisation of the studies on dispersion stability and found to be only moderately higher (ca. 10 %) than the bundle length). With single tube volume and the density determined for K-Nanos, the theoretical particle number per mg was calculated and used to determine the mass concentration required for obtaining a particle target concentration of 5.00E+12 for the study:

Calculation I			
	K-100	K-210	K-300
Bundle length, D50 [μm]	43.3	43.401	49.98
SingleTube diameter, D50 [μm]	0.01187	0.01208	0.01469
Volume single tube [μm^3]	0.004791585	0.004974203	0.0084709
Particles /cm^3	2.08699E+14	2.01037E+14	1.1805E+14
Relative density (g/cm^3)	1.761	1.761	1.761
Particles/g	1.18512E+14	1.14161E+14	6.7036E+13
Particles/mg	1.18512E+11	1.14161E+11	6.7036E+10
Target Concentration:			
5.00E+12 Particles/L			
Equivalent to [mg/L]	42.19	43.80	74.59
Study reports final, TG 318			
Mass conc. taken for the study [mg/L]	42.18	43.79	74.57
Particle conc. taken for the study [P/L]	5.00E+12	5.00E+12	5.00E+12

Please note: the theoretical particle concentrations associated with the mass concentrations used in the study are assumed to be <u>significant overestimates of the real particle concentration</u>. According to SEM and TEM images, K-Nanos (and Jenotubes) form bundles during synthesis (are 'entangled'), and the bundles further agglomerate to form higher order visually perceptible structures. Because it is not possible to (fully) separate MWCNTs entangled into bundles to single tubes by sonication without destruction of the single tubes, calculation of the particle concentration based on the dimensions of single tubes is assumed to significantly overestimate the actual particle concentration. <u>Because dispersion stability is inversely related to particle concentration, this setting will overestimate the dispersion stability.</u>

Performing the same calculation but <u>using D50 values for MWCNT bundle diameters</u>, resulting particle concentration corresponding to the mass concentrations used in respective studies are between

1.2E+08 and 3.43E+08 particles /L, i.e. particle concentrations used in the experiments would be 4 orders of magnitude lower than the target concentration of 5.00E+12 particles/L.
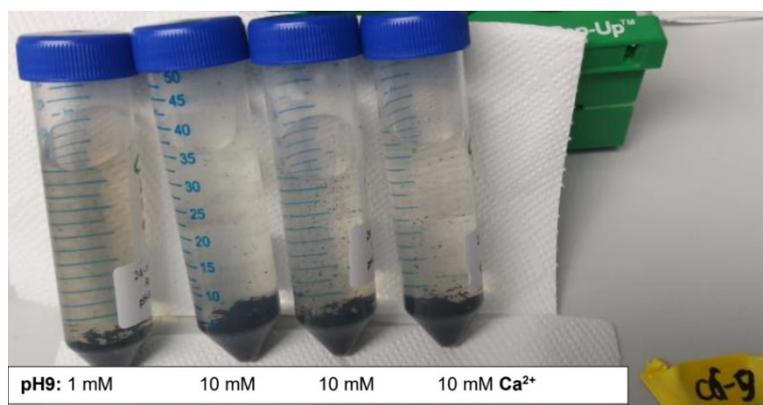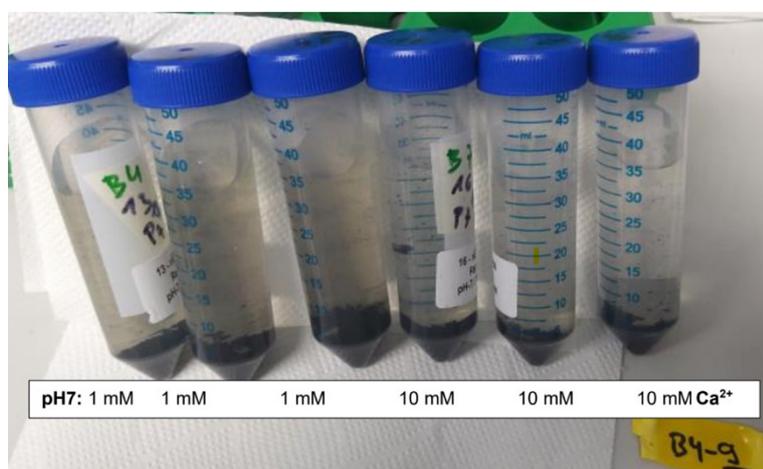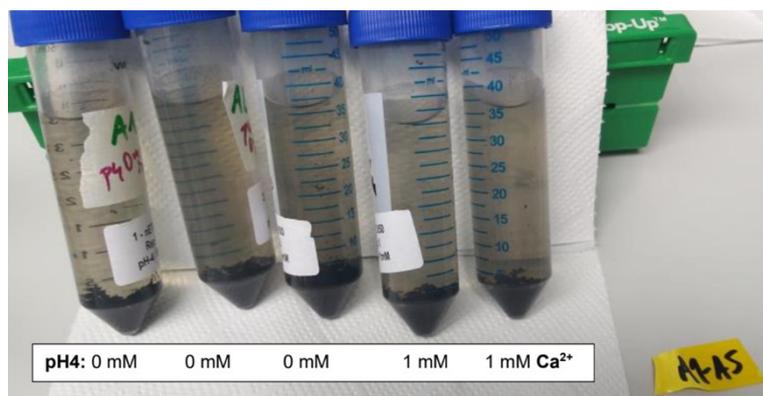

Figure S 1: Selected K-Nanos 100 MWCNT samples (pH 4, 7, 9, with various concentrations of electrolyte – 0, 1, 10 mM) after 6 h of incubation.

Image taken by nEcotox GmbH (2023), commissioned by KKPC.

Figure S 2: Dispersion of JENOTUBE 8 MWCNTs in pure water / stock dispersion

Stock dispersion of the test item and dilutions (8-0.5 mg/L). Fast agglomeration and inhomogeneous distribution are visible in the water column, even at very low concentrations.

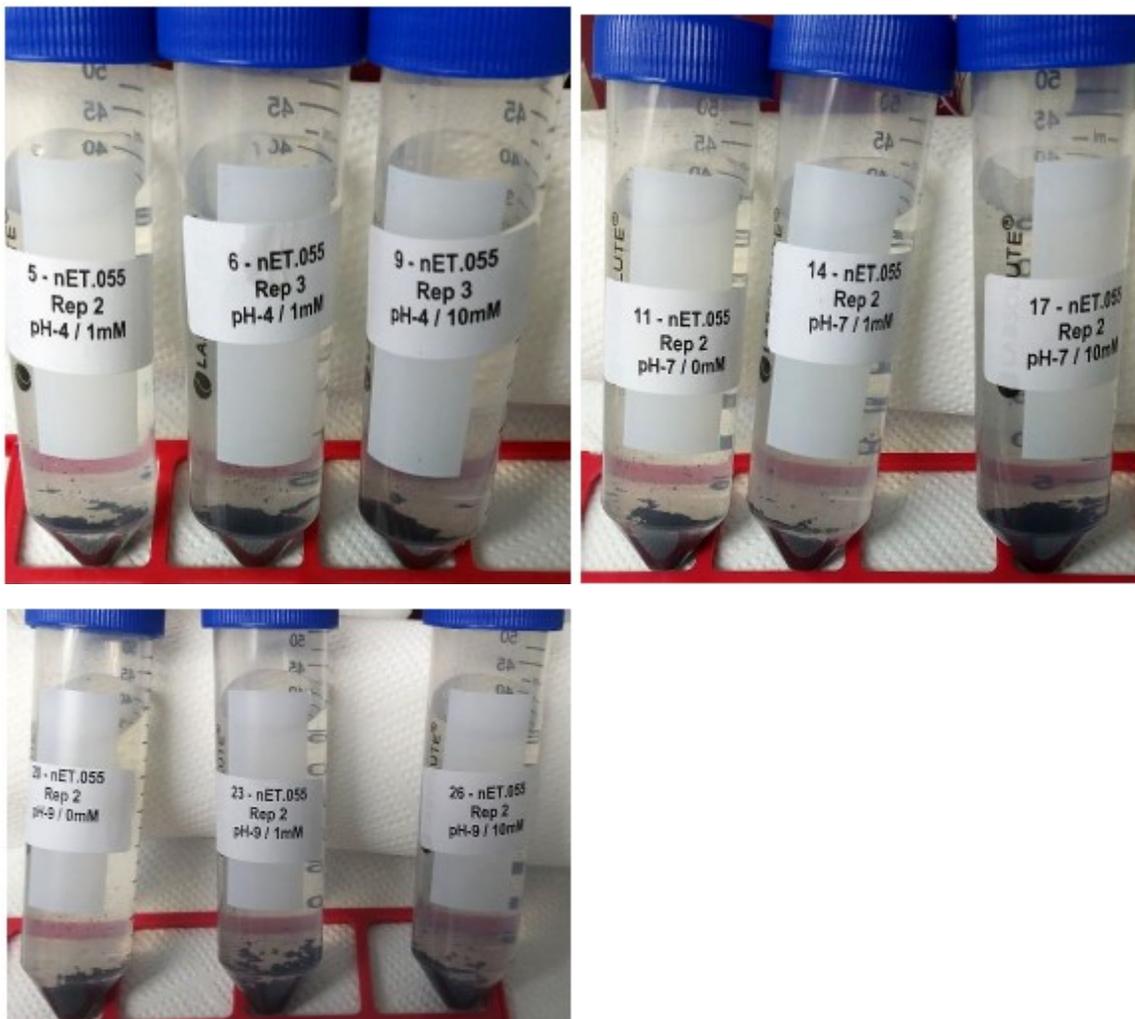*Image taken by nEcotox GmbH (2022), commissioned by JEIO.*

Figure S 3: Selected Jenotube 6 MWCNT samples (pH 4, 7, 9, with various concentrations of electrolyte – 0, 1, 10 mM) after 6 h of incubation.

Image taken by nEcotox GmbH (2024), commissioned by JEIO.

Table S 3: Method LODs and LOQs for UV-Visible spectrophotometer

Test material	Particle number conc. [particles/L]	Concentration [mg/L]	LOD [mg/L]	LOQ [mg/L]
K-Nanos 100	$5 \cdot 10^{12}$	42.18	0.25	1.07
K-Nanos 210	$5 \cdot 10^{12}$	43.79	0.54	2.01
K-Nanos 300	$5 \cdot 10^{12}$	74.57	0.22	0.66
Jenotube 6	$5 \cdot 10^{12}$	259.05	1.82	3.78
Jenotube 8	$3.5 \cdot 10^{11}$	2.95	0.19×10^{11} particles/L	0.31×10^{11} particles/L
Jenotube 10A	$5 \cdot 10^{12}$	344.14	1.11	3.13
Jenotube 10B	$5 \cdot 10^{12}$	542.17	1.51	4.17

4   DEGRADATION AND ADSORPTION/DESORPTION SCREENING
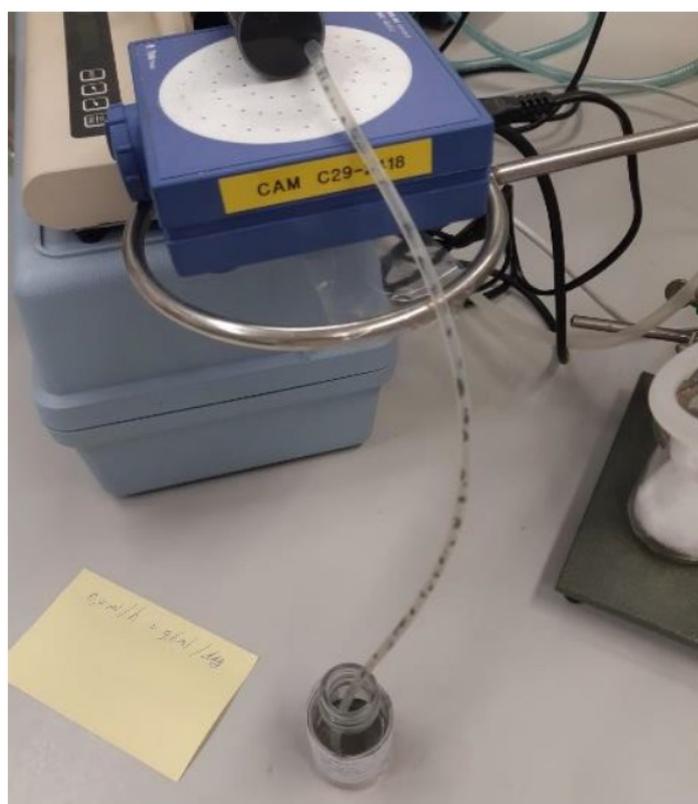
Figure S 4: Dosing unit of a Continuous Activated Sludge (CAS) reactor according to OECD TG 303 A.

Syringe filled with a mixture of 300 mg/L Jenotube 8 MWCNTs and 300 mg/L Tween 80 dosed into a bottle on the lab-table. Pump flow used (representative for CAS test) was 9.6 ml/day. The image shows MWCNT agglomerates and clogging in the tubing connected to the syringe.

Image taken by Nouryon Specialty Chemicals B.V. (2022), commissioned by TSafeE on behalf of JEIO.
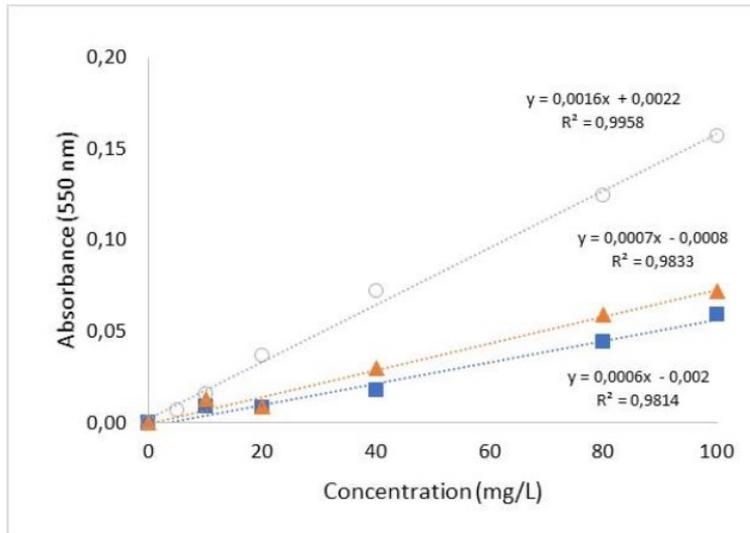
Figure S 5: Calibration curves of Jenotube 8 MWCNTs in demineralised water.

Three calibration curves of Jenotube 8 MWCNTs in demineralised water were prepared from two different stock suspensions (closed markers and open marker), which were visually evaluated as comparable with regard to colour and homogeneity of the mixture. Both stock suspensions contained 50 mg/L MWCNTs and 500 mg/L Tween 80 and were prepared identically. The calibration curves from the closed marker were prepared to reproduce the results of the calibration curve with the open markers.

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

OECD 303A Mass balance calculation						Copyright	29.07.2024
		Allowed range according to 303 A				Dr. Stefan Gartiser	
Volume activated sludge basin:	3 L	3 L activated slude + 1,5 secondary clarifier				Hydrotox GmbH, Freiburg	
Inflow per day:	12 L						
DOC synthetic sewage water:	100 mg/L						
DOC nano tubes:	20 mg/L	Normallly 10-20 mg/L DOC with upper concentration ≤ 50 mg/l.					
Acvtivated sludge concentration:	2,5 g/L	1-3 g/L					
Removal of surplus sludge per day:	0,375 L	depending on sludge age					
Sludge age:	8 days	6-10 days					
Adsorption to activted sludge:	99 %						
(as an example)							

Day	Cumulative dosage nano tubes	Removal surplus sludge	Cumulative removal of nano tubes with surplus sludge	Concentration nano tubes in activated sludge volume	Concentration of nano tubes in the activated sludge basin on a mass basis	Concentration of nano tubes in the outflow	Cumulative mass of nano tubes in the outflow
	mg	L/day	mg	mg/L	mg/g	mg/L	mg
1	240	0	0	80	32	0,2	2,4
2	480	0,375	30	148	59	0,2	4,8
3	720	0,375	56	219	88	0,2	7,2
4	960	0,375	82	289	116	0,2	9,6
5	1200	0,375	109	360	144	0,2	12
6	1440	0,375	135	430	172	0,2	14,4
7	1680	0,375	161	501	200	0,2	16,8
8	1920	0,375	188	571	228	0,2	19,2
9	2160	0,375	214	641	257	0,2	21,6
10	2400	0,375	241	712	285	0,2	24
11	2640	0,375	267	782	313	0,2	26,4
12	2880	0,375	293	853	341	0,2	28,8
13	3120	0,375	320	923	369	0,2	31,2
14	3360	0,375	346	993	397	0,2	33,6
15	3600	0,375	373	1064	426	0,2	36
16	3840	0,375	399	1134	454	0,2	38,4
17	4080	0,375	425	1205	482	0,2	40,8
18	4320	0,375	452	1275	510	0,2	43,2
19	4560	0,375	478	1345	538	0,2	45,6
20	4800	0,375	505	1416	566	0,2	48
21	5040	0,375	531	1486	594	0,2	50,4
Mass balance	5040	=	531	+	4459	+	50,4

Mass balance at the end of the test after 21 days				5040	mg nano tubes (absolute)		
			of which	11%	in surplus sludge		
			and	88%	in activated sludge basin		
			and	1%	in the outflow		
			Sum	100%			

Figure S 6: Example calculation illustrating the mass balance for MWCNTs under hypothetical OECD TG 303A test conditions.

Test conditions assumed for this model calculation include a MWCNT test concentration of 20 mg/L (continuously dosed to the CAS reactor) and an adsorption rate of 99 %.

The fate and behaviour of chemicals in Sewage Treatment Plants (STPs) are typically assessed using the Laboratory Sewage Treatment Plant Simulation Test (LSTP), as defined by OECD TG 303A. This test uses a synthetic organic wastewater as the primary carbon and energy source for the microorganisms. The test unit comprises an aeration vessel holding approximately 3 L of activated sludge and a separator (secondary clarifier) containing about 1.5 L of sludge. A typical setup involves running two units in parallel—one test unit and one reference unit—under identical conditions. Key parameters include a mean Hydraulic Retention Time (HRT) of 6 hours (based on a daily inflow of 12 L into the 3 L vessel) and a mean Sludge Age (Sludge Retention Time, SRT) of 6–10 days, determined by the removal of surplus sludge.

The test substance is added to the test unit (Unit 1) as a suspension. This condition allows for the determination of the distribution between the activated sludge and the effluent and the subsequent calculation of the overall mass balance.
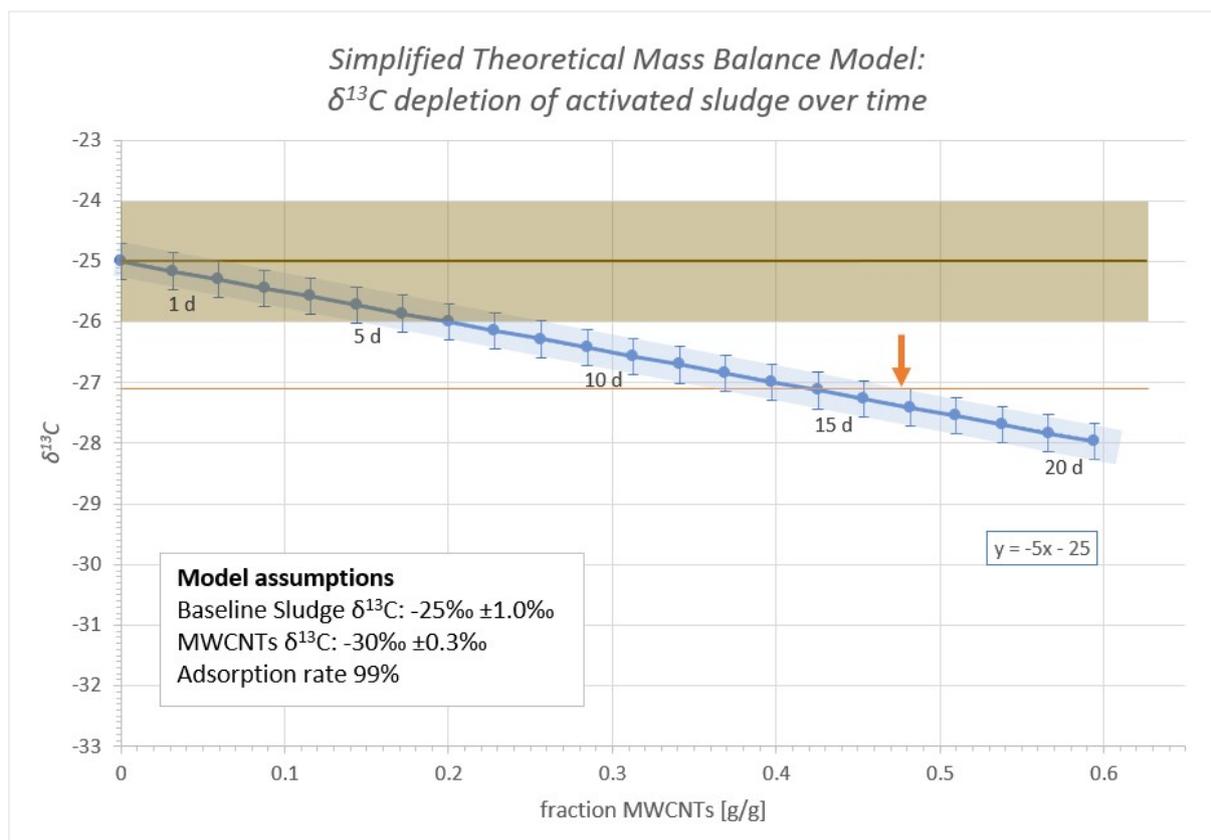
Figure S 7: Simplified theoretical mass balance model for an STP simulation study according to OECD 303A showing the time-dependent (days) isotopic shift (depletion) of $\delta^{13}C$ of the activated sludge in the CAS reactor by constant MWCNT dosing (20 mg/L) and an assumed 99 % adsorption rate. The total simulation period is 21 days.

Figure legend

➢ Brown line: $\delta^{13}C_{baseline}$ of activated sludge: -25 ‰
➢ Brown shaded area: $\delta^{13}C_{baseline} \pm 1.0$ ‰ (estimated SD) representing the 95 % Confidence Interval (CI). The estimated SD of 1.0 ‰ covers the variability found in available experimental baseline activated sludge data (UBA, 2022).
➢ Orange line: Least Significant Difference (LSD) threshold, $\delta^{13}C$ value required for statistical significance.
➢ Blue line: Isotopic shift (depletion) of $\delta^{13}C_{CAS}$
➢ Blue shaded area: Error bars for the $\delta^{13}C_{CAS}$ function, defined by the Standard Error of Measurement (SEM$_{meas}$) $= \pm 0.3$ ‰ (1σ).
➢ Orange arrow: Indicates the fraction of MWCNTs necessary for a statistically significant depletion of $\delta^{13}C_{CAS}$ from $\delta^{13}C_{baseline}$.

Model assumptions and explanation

➢ $\delta^{13}C$ Baseline sludge ($\delta^{13}C_{baseline}$): -25 ‰
➢ $\delta^{13}C$ Baseline Standard Deviation (SD$_{baseline}$): ±1.0 ‰, represents the realistic variability in the $\delta^{13}C$ of background sludge (e.g.,-24 ‰ to -26 ‰).
➢ $\delta^{13}C$ MWCNTs: -30 ‰, representative value for the test substance MWCNT (Jenotubes).
➢ $\delta^{13}C$ Standard error of Measurement (SEM$_{meas}$): ± 0.3 ‰, method precision (1σ).
➢ MWCNT adsorption rate: 99 % (example of an assumption).

Statistical Calculations (95 % CI)

The LSD is calculated by applying the two-sample t-test principle to the combined uncertainties of the Baseline Sludge (SD) and the MWCNT Analytical Precision (SEM), ensuring the minimum shift required for statistical significance is quantified. The minimum difference necessary to differentiate the two measurements is determined using the Standard Error of the Difference (SED):

$$SED = \sqrt{SD_{baseline}^2 + SEM_{meas}^2} = \sqrt{1.0\ ‰^2 + 0.3\ ‰^2} = 1.044\ ‰$$

The LSD is typically calculated for a conservative 95 % confidence interval (CI), which uses a critical t-value ($t_{critical} \approx 2$):

$$LSD = t_{critical} \times SED = 2 \text{ x } SED = 2 \times 1.044\ ‰ = 2.088\ ‰$$

The measured $\delta^{13}C_{CAS}$ must be at least 2.088 ‰ different from the $\delta^{13}C_{baseline}$ to be statistically significant.

LSD Threshold, is the depleted value that is 2.088 ‰ below the baseline:

$$LSD_{threshold} = \delta^{13}C_{baseline} - LSD = -25\ ‰ - 2.088\ ‰ = -27.088\ ‰$$

Mass Balance Calculation

The required MWCNT mass fraction (x) in the sludge is determined by the $LSD_{threshold}$ and SEM_{meas} ($Y_{(X)}$):

$$Y_{required} = \delta^{13}C_{CAS} = LSD_{threshold} - SEM_{meas} = -27.088\ ‰ - 0.3\ ‰ = -27.388$$

The derived function $Y_{(x)}$ of $\delta^{13}C_{CAS}$ is:

$$Y_{(x)} = \delta^{13}C_{CAS} = -5\ x - 25$$

$$x = \frac{Y_{required} - (-25)}{-5} = 0.4776 \approx 48\ \%$$

The required MWCNT mass fraction would be 48 %.

The plausibility of reaching high MWCNT mass fraction in the CAS reactor must be further evaluated, e.g. with regard to adsorption rates.

Table S 4: Published data of $\delta^{13}C$ isotopic signatures for STP sludges and SWCNTs.

Samples	$\delta^{13}C \pm SD$ [‰] [1]	Reference
$\delta^{13}C$ values for STP sludge		
Sludge solids in sewage – origin Japan	-24.8 ± 0.2	Ondera et al. (2015)
Sludge solids in effluent – origin Japan	-25.9 ± 0.1	Ondera et al. (2015)
Treated sewage – origin probably Eastern Europe	-28.5 to -21.0	Maksymowska et al. (2000) as cited in Ondera et al. (2015)
Sludge, wasted activated sludge, and biosolids (STP) – origin presumably Australia	-25.3 to -22.5	Law et al. (2013) cited in UBA[22]
Activated sludge – origin presumably Germany	-25.3; -25.1; -22.2	Hydroisotop proprietary database (2012), UBA (2022)
Activated sludge from 20 STPs – origin Germany	-24 ± 0.3 to -28 ± 0.3	UBA (2022)
$\delta^{13}C$ values for SWCNTs		
SWCNTs by CVD, feedstock ethylene (NanoAm)	-35.2	Plata et al. (2008)
SWCNTs by CVD, feedstock ethylene (SW-6)	-33.21	Chartrand et al. (2019)
SWCNTs by CVD (full range)	-55 to -31	Reported in Chartrand et al. (2019), Fig. 3
$\delta^{13}C$ values for MWCNTs		
MWCNTs, various synthesis methods (full range)	-44 to -27	Reported in Chartrand et al. (2019), Fig. 3
$\delta^{13}C$ values for MWCNTs		
K-Nanos 100	-29.2 ± 0.3	present study
K-Nanos 210	-29.0 ± 0.3	
K-Nanos 300	-29.4 ± 0.3	
Jenotube 6	-29.5 ± 0.3	
Jenotube 8	-30.6 ± 0.3	
Jenotube 10A	-27.4 ± 0.3	
Jenotube 10B	-27.9 ± 0.3	

[1] if reported.

ABBREVIATION

CAS – Continuous Activated Sludge

DLS – Dynamic Light Scattering

MWCNTs – Multi-Walled Carbon Nanotubes

LSTP – Laboratory Sewage Treatment Plant

LSD – Least Significant Difference

LOD – Limit of Detection

LOQ – Limit of Quantification

PVP – Polyvinylpyrrolidone

SD – Standard Deviation

SED – Standard Error of Difference

SEM – Standard Error of Measurement

SWCNTs – Single-Walled Carbon Nanotubes

REFERENCES

T. Onodera, G. Kanaya, K. Syutsubo, Y. Miyaoka, M. Hatamoto and T. Yamaguchi, Spatial changes in carbon and nitrogen stable isotope ratios of sludge and associated organisms in a biological sewage treatment system, *Water Research*, 2015, **68**, 387-393.

D. L. Plata, P. M. Gschwend and C. M. Reddy, Industrially synthesized single-walled carbon nanotubes: compositional data for users, environmental risk assessments, and source apportionment, *Nanotechnology*, 2008, **19**, 185706.

UBA, Umweltbundesamt, Bestimmung der biogenen Kohlenstoffgehalte von Klärschlamm und Faulgas und Untersuchung von Abhängigkeiten zu Kläranlagen-Basisdaten, Abwasserwerten und Klärschlammzusammensetzung, TEXTE 149/2022, Dessau-Roßlau, Germany, 2022.

M. M. G. Chartrand, C. T. Kingston, B. Simard and Z. Mester, Carbon Isotopic Measurements of Nanotubes to Differentiate Carbon Sources, *ACS Omega*, 2019, **4**, 22108-22113.