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NANOMATERIALS

TiO_2 nanoparticles – Relationship between dispersion preparation method and ecotoxicity in the algal growth test

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Abstract *Purpose* Little is known about the ecotoxicity of nanomaterials and there are no specific guidelines for sample preparation and testing. We set out to establish whether the method used to prepare TiO_2 dispersions had a significant impact on aquatic ecotoxicity. We also followed the formation of agglomerates during the incubation period.

Methods We applied the algal growth inhibition test (OECD test guideline no. 201). Dispersions were prepared by stirring and/or ultrasonication for different durations, and by filtration according to an OECD procedure recommended for testing difficult substances.

Results Samples stirred for 7 d were not toxic, but EC_{20} values could be calculated for all the other treatments. Shorter treatments generated EC_{20} values in the range 1–27 mg/L. Only the shortest treatment (1 min stirring, 1 min ultrasonication) produced an unusually high EC_{20} value, indicating low toxicity. Development of agglomerate size and of toxicity depends on the nanoparticles. We found that ecotoxicity was predominantly caused by a fraction of nanoparticles and agglomerates obtained by passing dispersions through a 0.22-µm filter.

Conclusions We propose a short treatment regime to generate the most relevant ecotoxicity data for TiO_2, for example stirring for 1 min followed by 3 min ultrasonication. Until more data concerning the ecotoxicity of different fractions are available, we recommend the testing of unfiltered dispersions rather than filtrates. Relating ecotoxicity to the total hydrodynamic surface of the nanomaterials rather than concentration does not seem to improve the accuracy of ecotoxicity assessments using the algal growth inhibition test.

Keywords TiO_2 nanoparticles · Dispersion preparation · Ecotoxicity · Green algae

1 Introduction

Little is known about the ecotoxicity of nanomaterials and there are no specific guidelines for sample preparation and testing. Since the bioavailability and toxicity of nanomaterials may depend on the preparation method, meaningful comparisons between different studies can be difficult to achieve. ISO 14442 (2006) and the OECD series on testing and assessment no. 23 (2000) provide guidelines for the preparation of insoluble materials, including methods such as stirring (from several hours up to 6 weeks), ultrasonication, high-shear mixing, the addition of solvents or emulsifying agents, and the removal of non-dissolved test substances by filtration or centrifugation. However, neither document refers specifically to nanomaterials.

Several procedures for the specific testing of nanomaterials have been described in the literature, and whereas some authors do consider environmental relevance, others simulate a worst case scenario by striving for maximum dispersion. The methods include stirring for varying time periods, ultrasonic dispersion or the use of organic solvents for metal oxides, sometimes followed by filtration. For example, Lovern and Klaper (2006) suspended TiO_2 and fullerenes in water by sonication for at least 30 min and in some cases used tetrahydrofuran (THF) as a solvent, Lin and Xing (2007) sonicated their samples for 30 min and prevented aggregation by continuous stirring until the dispersion was used, Linkous et al. (2000) stirred for 12 h followed by vacuum

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filtration, Adams et al. (2006) used vigorous shaking of a stock dispersion, Beck-Speier et al. (2001) vortexed the samples five times for 3 s, and Oberdörster et al. (2006) stirred a stock solution for at least two months.

Among the three crystalline polymorphic phases of TiO_2, anatase has the highest photocatalytic activity and is therefore the most effective and widely used photocatalyst (Choi et al. 2004). Ecotoxicity may vary according to the efficiency of dispersion, reflecting the panel of different methods described above. For example, the toxicity of dispersions containing a large number of photocatalytically active particles may differ from that of filtrates containing only small particles or agglomerates but with higher bioavailability.

We set out to establish whether the method used to prepare TiO_2 dispersions had a significant impact on aquatic ecotoxicity as determined by the algal growth inhibition test (OECD test guideline no. 201 2006). We also followed the formation of agglomerates during the incubation period and assessed its impact on toxicity.

As the OECD guideline concerning algae toxicity testing should be applied without amending the test medium further, dispersions were prepared using the most common methods, i. e. stirring alone (1 min to 7 days), ultrasonication alone (1–15 min), a combination of these treatments (1 min to 3 days stirring followed by 1–15 min ultrasonication) and filtration according to the OECD test guidelines. The test procedure comprising 1 min stirring and 3 min ultrasonication was used to determine the effect of agglomerates on toxicity. We present our recommendations concerning the testing procedure that should be used to determine the ecotoxicity of nanomaterials.

2 Materials and methods

2.1 Description of TiO_2

Two TiO_2 materials were studied, one with a primary particle size of 8 nm (BET 250 m^2/g; Fig. 1; described hereafter as "small particles") and another with a primary particle size of 150 nm (BET 8 m^2/g; Fig. 2; described hereafter as "large particles"). Each was produced by an industrial partner using the sulfate process resulting in >90 % purity (the main impurities were iron, sulfate, adsorbed carbon species, and water). Heavy metals such as arsenic, mercury, and lead were below the toxicity levels stipulated by EFSA for the use of substances as a food and feed additive (E171).

2.2 Test dispersions

Test dispersions of the two materials were prepared at different concentrations by weighing the appropriate amount of TiO_2 on a Mettler AT261 Delta Range Semi-Micro Balance (range 0.01 mg to 62 g). The dispersion was prepared in 500 mL sterilized algae test medium in glass bottles according to OECD test guideline no. 201. Test concentrations ranged from 3.7–100 mg/L and differed by a separation factor of 1.5 or 3 mg/L. The bottles were covered with aluminum foil to prevent photocatalytic reactions. The nanomaterials were suspended by stirring with magnetic fleas (300 rpm) and/or ultrasonication in a bath sonicator filled to one third of the dispersion height in the bottles (Bandelin Sonorex RK 514 BH; 35 kHz; 215/860 W).

Three sets of parameters were tested, as listed below:

Experiment A (wide range of stirring parameters, with or without ultrasonication)

- 1 min stirring, 15 min ultrasonication
- 15 min stirring
- 1 day stirring

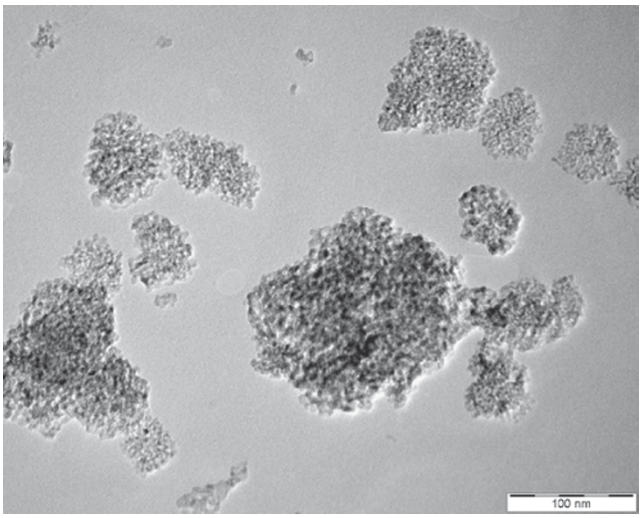

Fig. 1 TEM image of the smaller TiO_2 nanoparticles

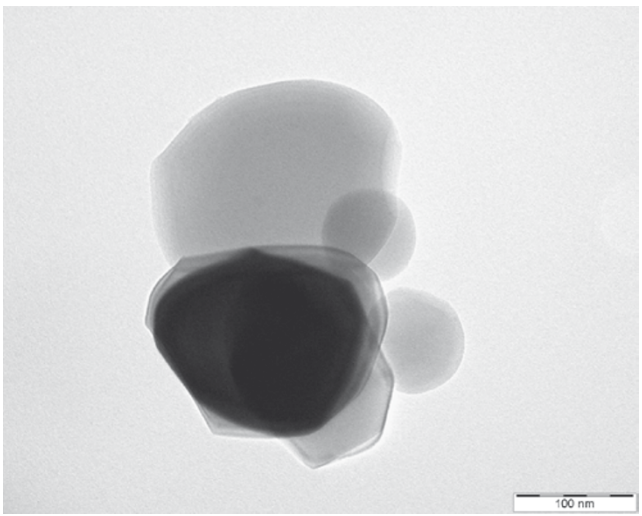

Fig. 2 TEM image of the larger TiO_2 nanoparticles

- 3 days stirring, 15 min ultrasonication
- 7 days stirring

Experiment B (stirring for 1 min followed by narrow range of ultrasonication parameters)

- 1 min stirring, 1 min ultrasonication
- 1 min stirring, 3 min ultrasonication
- 1 min stirring, 7 min ultrasonication
- 1 min stirring, 15 min ultrasonication

Experiment C (narrow range of stirring parameters followed by ultrasonication for 3 min)

- 1 min stirring, 3 min ultrasonication
- 3 min stirring, 3 min ultrasonication
- 7 min stirring, 3 min ultrasonication
- 15 min stirring, 3 min ultrasonication

For experiments investigating the shape of the concentration-effect curve and agglomerate formation, stirring was carried out for 1 min followed by sonication for 3 min.

2.3 Test solutions according to „Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures“ (OECD test guidance no. 23, 2000)

We added 200 mg of the TiO_2 sample material to 2 L growth medium under sterile conditions in a 2 L brown glass flask with a drain port. The preparation was stirred vigorously (700 rpm) at room temperature for 48 h. Non-dissolved solids were then removed by passing the dispersion through a 0.22 µm-filter (Millipore MCE-MF, cellulose nitrate and acetyl cellulose, no. GSWP04700) without pre-filtration. The clear filtrate, representing the highest test concentration, was diluted with growth medium to prepare further test concentrations differing by a separation factor of 2.

2.4 Chemical analysis of test solutions according to OECD test guidance no. 23

Samples were acidified with concentrated Suprapur® nitric acid (MERC) to a final concentration of 1 % (v/v) immediately after sampling, a treatment recommended to stabilize metal solutions. The total Ti content was then determined using collision cell inductively coupled plasma mass spectrometry (ICP-MS) according to DIN 38406-29 (1999).

The calibration function was calculated using the linear regression algorithm supplied with the ICP-MS instrument software. The limits were calculated from the calibration curve following DIN 32645 (1994). The LOD (limit of detection) provided by the software was tripled to obtain the LOQ (limit of quantification). Test item concentration was measured as total Ti (isotope mass 49, tune #1 in no gas modus). The correlation coefficient was ≥0.999; LOD in the aqueous sample 0.02 µg/L; LOQ in the aqueous sample 0.06 µg/L; linearity range (analyte solution preparation for ICP-OES) 0.1–100 µg/L.

The certified reference waters TMDA-54.4 and TM-28.3 (obtained from Environment Canada) were analyzed as quality assurance samples along with the algal growth medium after filtration (test medium). Total Ti levels in these samples were below the LOD. The Ti recovery of the certified reference waters was 93.9–106 %. In order to test the recovery of Ti from the test medium, representative spiked samples were prepared (5 ml medium spiked with 1 mL of Ti standard, 250 µg Ti/L) and analyzed by collision cell ICP-MS as described above. The data are summarized in Table 1.

2.5 Agglomerate characterization

Agglomeration was characterized immediately after preparing the dispersions by analyzing the distribution of surface and particle sizes using a Malvern Mastersizer 2000 particle size analyzer coupled with a dispersion unit Hydro 2000 MU. A refraction index of 2.55 was applied. To avoid modifying agglomerate sizes, dispersions were not diluted prior to measurement. The data were presented as specific surface area (hydrodynamic surface) and as the d(0.10) value, which is part of the size distribution curve and indicates the upper diameter of the smallest 10 % of particles and agglomerates in the dispersion. In selected samples, the zeta potential was measured using a Malvern Zeta Sizer Nano ZS.

2.6 Ecotoxicological tests

The ecotoxicity of the dispersions was determined using an algal growth test according to OECD test guideline no. 201. The test species was *Pseudokirchneriella subcapitata* (obtained from the Culture Collection of Algae at the University of Göttingen, Germany; SAG database no. 61.81). Three days prior to testing, a pre-culture was established in sterile OECD growth medium according to test guideline no. 201 to obtain exponentially growing algae. All stock so-

Table 1 Recovery measurements using the test medium

Replicate no.	Measured conc. Level [µg Ti/L]	Fortification, final conc. [µg Ti/L]	End conc. [µg Ti/L]	Measured [µg Ti/L]	Recovery [%]
Sample 2/1	4.00	41.0	45.0	42.6	94.7
Sample 3/1	6.50	40.6	47.1	42.1	89.4

lutions for the OECD medium were prepared with purified water processed using an ELGA "PURELAB Ultra". Cell concentrations were calculated using an electronic particle counter (CASY 1 Model TT, Schärfe System, Reutlingen, Germany).

Tests dealing with dispersion preparation methods were performed in 96-well microtiter plates following test guideline ISO 8692 (Hund-Rinke and Simon 2006) using six replicates per test concentration. This allowed more tests to be carried out in parallel, improving the comparison of test results. Each well was filled with 180 µL of test medium and 20 µL of pre-culture (10^5 cells/mL) to achieve an initial cell density of 10^4 cells/mL.

Tests dealing with agglomeration were carried out in 250 mL Erlenmeyer flasks covered with silicone-sponge caps according to OECD test guideline no. 201, using four replicates per test concentration. Each flask was filled with 100 mL of test medium and enough pre-culture was added to achieve an initial cell density of 10^5 cells/mL. The larger volume provided enough dispersion to characterize the agglomerates. In selected experiments additional vessels were prepared to screen agglomerate sizes, and these lacked algae to avoid interference with particle size determination.

All experiments were incubated at 22 ± 1 °C with light intensity adjusted to ~7000 lux (95 µE m^{-2} s^{-1}) provided by OSRAM L 36 W/21-840 Plus Eco lamps. The light intensity was measured using an LI-189 luminance meter with radiation sensor (LI-COR, Lincoln, USA) with a cosine (2π) receptor in lux. The cultures were kept in suspension by rotary shaking at 100 rpm on a Multitron Incubation Shaker (INFORS, Switzerland).

For the dispersions in microtiter plates, algal biomass was determined after 0, 24, 48, and 72 h by recording the fluorescence intensity using a Tecan Spectrafluorplus microtiter plate reader. The same approach was used for the experiments with dispersions carried out in Erlenmeyer flasks, by transferring 200 µL aliquots to microtiter plates prior to measurement. The pH of the Erlenmeyer-flask cultures was tested at the beginning and end of each test using additional replicates.

2.7 Evaluation of the results

Evaluations of concentration-effect relationships and calculations of effective concentrations were based on the nominal concentrations of the test media, and on the mean values for fluorescence or cell number for each concentration. The percent inhibition of growth rate [r] and yield [y] were calculated according to the OECD test guideline no. 201. EC_{20} and EC_{50} values were determined with 95 % confidence intervals by Probit analysis (Finney 1984) assuming log-normal distribution of the values using ToxRat® Professional 2.10.

3 Results

3.1 Preparation of test medium – testing particle dispersion methods

Table 2 presents data for the first TiO_2 sample (smaller particles; see Fig. 1) including the EC_{20} (growth rate) and the specific surface areas and d(0.10) values for the agglomerates, all presented as means for different test concentrations. As stated above, three experiments were carried out with different stirring and ultrasonication parameters: experiment A with wide-ranging parameters, experiment B with a fixed stirring duration and variable ultrasonication, and experiment C with variable stirring duration and fixed ultrasonication. Samples stirred for 7 d were not toxic, but EC_{20} values could be calculated for all the other treatments. Shorter treatments, i. e. stirring for up to 3 d followed by up to 15 min of ultrasonication generated EC_{20} values in the range 1–27 mg/L. Only the shortest treatment (1 min stirring, 1 min ultrasonication) produced an unusually high EC_{20} value, indicating low toxicity. Ultrasonication increased the specific surface area (and reduced the d(0.10) value) by promoting disaggregation more efficiently and breaking up the large agglomerates formed by stirring, since the specific surface areas were lowest and d(0.10) values highest in the three tests without an ultrasonication step. However, experiment B showed that 1 min of ultrasonication was sufficient to maximize the specific surface area and longer durations had no additional effect, whereas experiment C showed that the duration of stirring prior to ultrasonication also had no effect. There appeared to be no clear relationship between specific surface area and toxicity. The results were very similar for the second TiO_2 sample (larger particles; see Fig. 2) and are not presented here.

3.2 Preparation of test medium – testing filtrates derived from particle dispersions

The toxicity of filtrates derived from particle dispersions is summarized in Table 3, which shows the percentage inhibition of growth rate and biomass yield according to the nominal concentration of the test items. Significant inhibition of algal growth was observed only at the highest concentration (100 mg/L), which resulted in a 51.0 % reduction in growth rate and a 92.3 % reduction in yield. We calculated an EC_{50} (growth rate) value of 99.0 mg/L (confidence interval: 96.9–101.4), and an EC_{50} (yield) value of 65.8 mg/L (confidence interval: 60.7–72.6). Microscopy showed that the algae were morphologically normal even though the amount of cell debris in the cultures increased in line with growth inhibition. No particles or agglomerates were detected in the test media using the Mastersizer 2000, although chemical analysis identified small amounts of Ti (10–109 µg/L,

Table 2 Growth test with *Pseudokirchneriella subcapitata* – different dispersion methods and corresponding EC_{20} values (growth rate) with confidence intervals for the smaller TiO_2 nanoparticles

Treatments for dispersion preparation					
Stirring	**Ultrasonication**	**Growth rate EC_{20} [mg/L]**	**95 % confidence interval**	**Specific surface area [m^2/g]**	**d(0.10) [µm]**
Experiment A: wide variation of stirring and ultrasonication					
1 min	15 min	16.1	14.0–18.6	3.7	0.87
15 min	–	10.6	6.6–16.3	3.0	1.13
1 d	–	22.7	20.0–25.3	2.9	1.14
3 d	15 min	4.3	3.7–4.9	3.9	0.82
7 d	–	No toxicity	–	2.9	1.05
Experiment B: fixed stirring period, variation of ultrasonication					
1 min	1 min	46.0	34.4–65.3	3.6	0.87
1 min	3 min	25.5	11.8–57.6	3.7	0.85
1 min	7 min	22.2	16.0–29.4	3.3	0.92
1 min	15 min	27.2	18.2–40.6	3.8	0.85
Experiment C: variation of stirring, fixed ultrasonication period					
1 min	3 min	6.9	2.8–11.4	3.7	0.85
3 min	3 min	8.9	2.8–16.3	3.7	0.83
7 min	3 min	4.0	2.0–6.3	3.6	0.82
15 min	3 min	0.8	0.1–2.4	3.7	0.82

Table 3 Percent inhibition of growth rate and yield, and EC_{50} values for a 72-h incubation (smaller nanoparticles; testing of filtrates)

Test item nominal concentration [mg/L]	Inhibition of growth rate [%]	Inhibition of yield [%]
6.25	1.1	5.4
12.5	1.2	5.5
25.0	2.1	9.9
50.0	3.9	17.5
100	51.0	92.3
EC_{50} [mg/L] (95 % confidence interval)	65.8 (60.7–72.6)	99.0 (96.9–101)

Table 4 Total Ti concentrations at the beginning of test with filtrates

Nominal concentration		Measured after filtration at test start
[mg TiO_2/L]	**[mg Ti/L]**	**[µg Ti/L]**
Control	–	0.08
6.25	3.75	9.67
12.5	7.49	19.2
25.0	14.99	31.4
50.0	29.98	53.1
100	59.95	109

equivalent to 16.7–167 µg TiO_2/L) revealing that some TiO_2 particles had passed through the 0.22 µm filter (Table 4).

3.3 Testing particle dispersions – concentration-effect curve and agglomerate formation

Figures 3 and 4 show the concentration-effect curves for the small TiO_2 particles, reflecting the impact on growth rate and yield, respectively (dispersion prepared by stirring for 1 min followed by sonication for 3 min). The results of the four replicates per test concentration were comparable, in most cases varying by <10 %. Corresponding fluorescence data are presented in Table 5.

Toxicity increased in line with concentration up to 44 mg/L. After a 24-h incubation, toxicity reached a plateau at this concentration, indicating that additional TiO_2 had no further toxic effect. There was a maximum 15 % inhibition of growth rate and 20 % inhibition of yield. The profile was similar for the 72-h incubation, although in this case test concentrations above 44 mg/L declined slightly in toxicity. There was a maximum 40 % inhibition of growth rate and 70 % inhibition of yield, which is more pronounced than the maxima at 24 h. EC values for yield and growth, for both the 24 and 72 h incubations, are presented in Table 6. EC_{20} values for yield ranged from 51.5 mg/L (24 h) to 6.4 mg/L (72 h), whereas growth rate inhibition was below 20 % after 24 h and reached an EC_{20} value of 16.5 mg/L after 72 h.

At the beginning of the incubation period, the specific surface area of the agglomerates was comparable in each of the different test concentrations. However, the size of agglomerates increased over the next 72 h resulting in a decline in the specific surface area (Fig. 5) and a concomitant increase in the corresponding d(0.10) values (Fig. 6). The increase was more pronounced at the lower

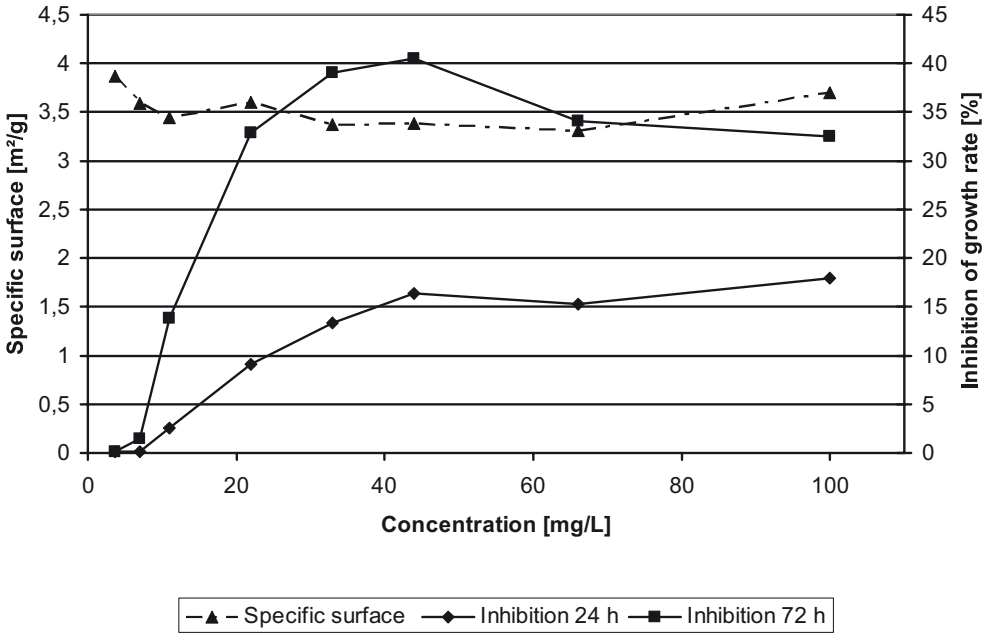

Fig. 3 Concentration-effect curve (growth rate) and specific surface area of the smaller TiO_2 nanoparticles

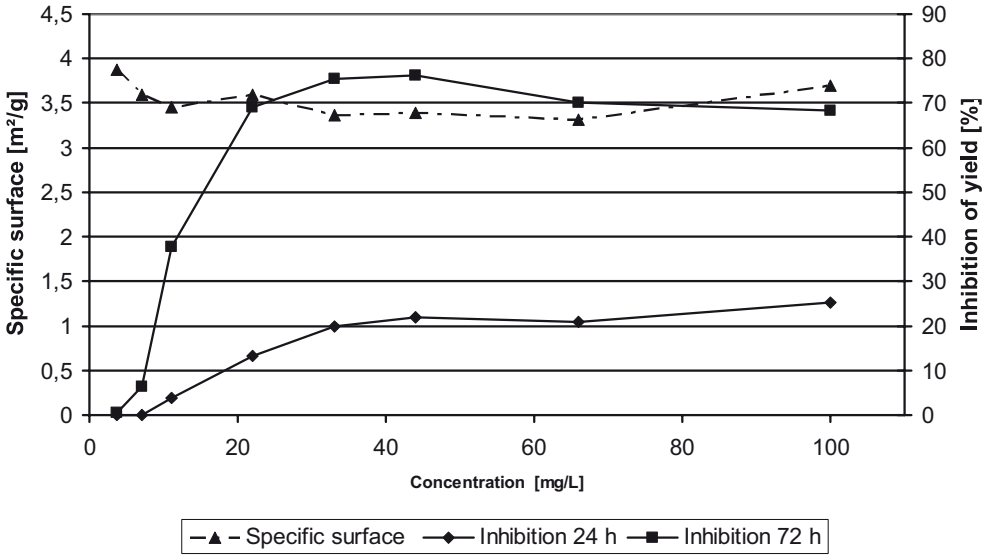

Fig. 4 Concentration-effect curve (yield) and specific surface area of the smaller TiO_2 nanoparticles

test concentrations. The mean specific surface area of all test concentrations was initially 3.54 ± 0.28 m²/g decreasing to 0.62 ± 0.63 m²/g after 72 h, the higher standard deviation reflecting the concentration-dependent extent of agglomerate formation. Microscopy showed that TiO_2 agglomerates were attached to algal cells, obscuring potential cell debris. The loading of algae with agglomerates reflected the amount of TiO_2 in the test.

In contrast to the small TiO_2 particles, the toxicity of the large TiO_2 particles decreased during the incubation period (Fig. 7), with a maximum 72 % inhibition of growth rate at 66 mg/L after 24 h, falling to a maximum 54 % inhibition (this at the highest concentration of 100 mg/L, the value at 66 mg/L was 42 %) after 72 h. The effect on yield was more complex (Fig. 8). The cells were more strongly inhibited in the short term at lower concentrations, but appeared to recover by 72 h, whereas the cells were affected to approximately the same extent for both incubation periods at the highest concentrations. The EC values for the large TiO_2 particles are presented in Table 6. EC_{20} values for yield ranged from 1.0 mg/L (24 h) to 8.6 mg/L (72 h), and for growth rate they ranged from 1.9 mg/L (24 h) to 29.8 mg/L (72 h).

The specific surface area of the large TiO_2 particles exceeded that of the small particles by approximately threefold. The specific surface area of the large particles decreased during the incubation period and the d(0.10) value increased. In contrast to the small particles, the changes oc-

Table 5 Fluorescence raw data for the smaller TiO_2 nanoparticles dispersed by 1 min stirring followed by 3 min ultrasonication

	TiO2 concentration [mg/L]								
	0 (Control)	**3.7**	**7**	**11**	**22**	**33**	**44**	**66**	**100**
0 h									
Mean value	233.0	228.3	229.2	233.2	234.2	231.8	238.8	237.5	235.0
Standard deviation	5.7	6.0	5.1	4.6	4.7	4.5	1.6	8.0	6.1
Variance [%]	2.5	2.6	2.2	2.0	2.0	2.0	0.7	3.4	2.6
24 h									
Mean value	612.7	621.5	619.2	598.0	563.8	535.5	535.5	537.8	518.5
Standard deviation	41.8	17.1	22.1	24.2	32.5	20.9	18.0	24.1	10.0
Variance [%]	6.8	2.8	3.6	4.0	5.8	3.9	3.4	4.5	1.9
48 h									
Mean value	1829.8	1860.3	1623.3	1407.0	1190.2	905.3	819.0	933.2	965.5
Standard deviation	268.2	100.6	59.1	112.2	378.2	50.8	20.9	46.1	47.8
Variance [%]	14.7	5.4	3.6	8.0	31.8	5.6	2.6	4.9	5.0
72 h									
Mean value	6630.7	6587.8	6219.7	4221.5	2211.5	1783.3	1756.3	2153.3	2257.5
Standard deviation	495.9	356.0	195.3	667.8	119.0	128.6	142.8	87.4	224.7
Variance [%]	7.5	5.4	3.1	15.8	5.4	7.2	8.1	4.1	10.0

Table 6 EC values (and 95 % confidence interval in parentheses) for both types of TiO_2 nanoparticles dispersed by 1 min stirring followed by 3 min ultrasonication

EC-values	**Smaller TiO_2 particles**		**Larger TiO_2 particles**	
	Growth rate	**Yield**	**Growth rate**	**Yield**
EC_{20}–24 h [mg/L]	Highest inhibition below 20 %	51.5 (43.2–62.1)	1.9 (0.9–2.2)	1.0 (0.4–1.8)
EC_{50}–24 h [mg/L]	Highest inhibition below 50 %	Highest inhibition below 50 %	18.4 (14.3–23.1)	10.8 (7.8–14.0)
EC_{20}–72 h [mg/L]	16.5 (11.1–21.6)	6.4 (4.4–8.3)	29.8 (27.5–31.9)	8.6 (7.0–10.1)
EC_{50}–72 h [mg/L]	Highest inhibition below 50 %	19.7 (16.4–23.3)	Highest inhibition below 50 %	31.8 (28.8–35.2)

curred mainly during the first 24 h at all test concentrations. The mean specific surface area of all test concentrations was initially 10.17 ± 1.01 m²/g decreasing to 0.88 ± 0.43 m²/g after 72 h. The final values for the large TiO_2 particles were of the same order of magnitude as for the small particles.

The pH values and zeta potentials of the tests are presented in Tables 7 and 8. The pH increased during incubation due to algal growth. The zeta potential of both the algae and the TiO_2 nanoparticles was negative at the start of the incubation, and in the case of the control sample lacking algae this parameter did not change. The zeta potential in the control containing algae alone became more negative during the incubation period, and this trend was repeated in experimental tests with algae and low concentrations of nanoparticles. In contrast, the experiments with algae and higher concentrations of nanoparticles showed no clear trend. No clear difference in the characteristics of the large and small nanoparticles was observed.

4 Discussion

4.1 Testing dispersions

4.1.1 Dispersion preparation methods

The influence of preparation method on the toxicity of nanomaterial-containing dispersions appears to be small, unless long-term preparation methods are applied. Stirring for 7 days resulted in no toxicity up to the highest test concentration, but EC_{20} values could be calculated for the other treatments although there seemed to be no clear relationship between the method used and the resulting toxicity (see Table 2). Indeed, there were two pairs of tests in which identical conditions were applied in different experiments. The results indicate that variability between experiments was more pronounced than the variability between the different treatments: 1 min stirring and 15 min ultrasonication gave

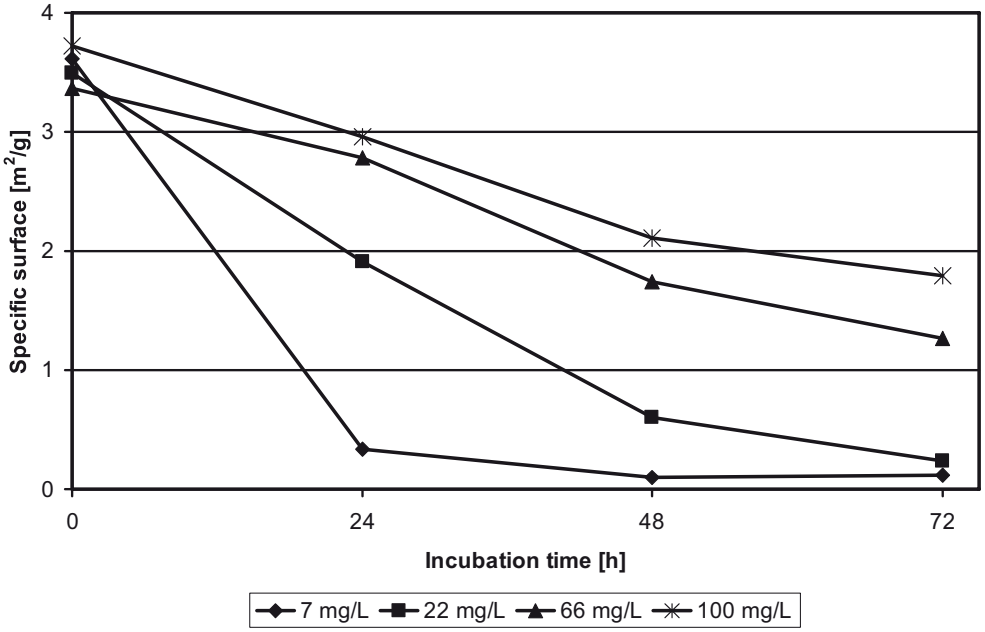

Fig. 5 Agglomerate formation (specific surface area) of the smaller TiO_2 nanoparticles under the conditions used in the algae test

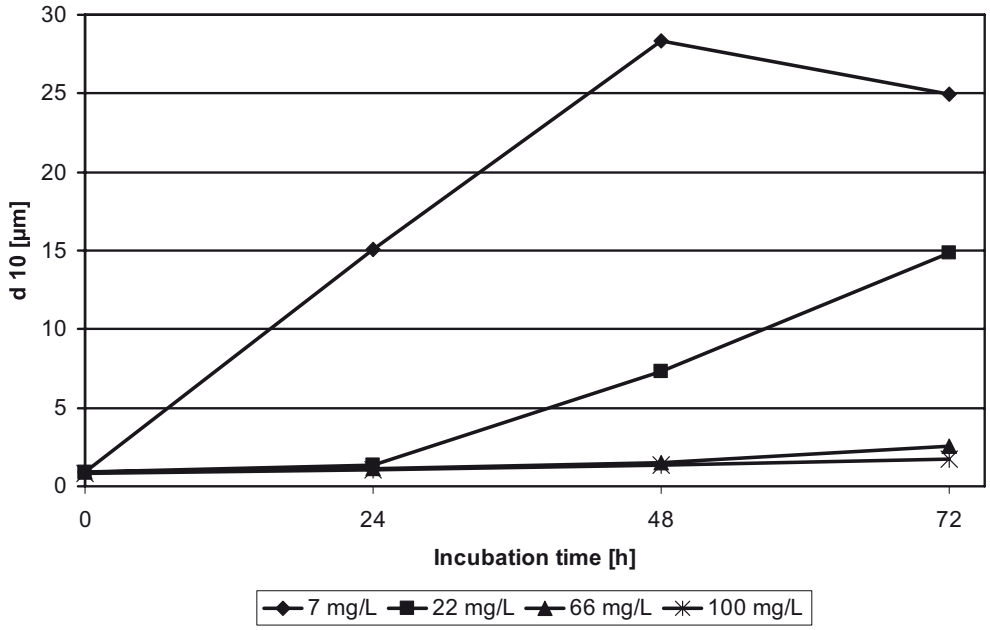

Fig. 6 Agglomerate formation (d(0.10) value) of the smaller TiO_2 nanoparticles under the conditions used in the algae test

EC_{20} values of 16.1 mg/L and 27.2 mg/L in experiments A and B, whereas 1 min stirring and 3 min ultrasonication gave EC_{20} values of 25.5 mg/L and 8.9 mg/L in Experiments B and C. The parameters characterizing the agglomerate size (surface, d(0.10)) were comparable or even identical for the pairs of tests. Therefore, it seems that these parameters indicate toxicity only roughly.

Ultrasonication had an impact on the specific surface area of the dispersions but there was no obvious impact on toxicity. The absence of correlation might be explained by agglomeration, resulting in changes to the specific surface

Table 7 pH measurements for selected concentrations (test with dispersions performed in Erlenmeyer flasks)

	TiO_2 concentration [mg/L]				
	0 (Control	**7**	**22**	**66**	**100**
	Smaller particles				
Day 0 (test start)	7.7	7.6	7.6	7.5	7.5
Day 3 (test end)	8.0	8.1	8.0	8.0	8.0
	Larger particles				
Day 0 (test start)	7.7	7.7	7.7	7.7	7.7
Day 3 (test end)	8.0	8.3	8.2	8.0	8.1

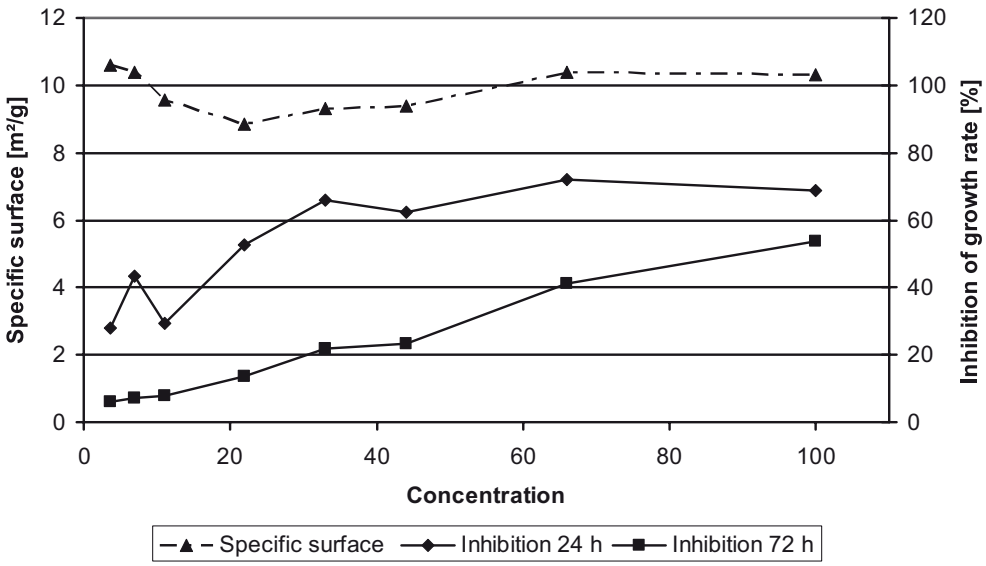

Fig. 7 Concentration-effect curve (growth rate) and specific surface area of the larger TiO_2 nanoparticles

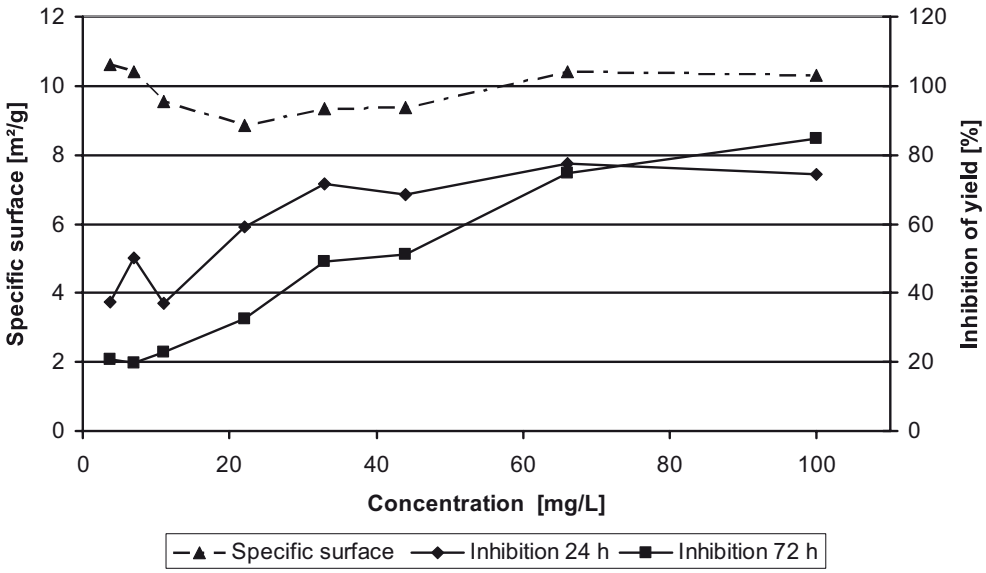

Fig. 8 Concentration-effect curve (yield) and specific surface area of the larger TiO_2 nanoparticles

Table 8 Zeta potential measurements [mV] for selected concentrations (test with dispersions performed in Erlenmeyer flasks)

	TiO_2 concentration [mg/L]								
	0 (Control)	**7**		**22**		**66**		**100**	
	With algae	**Without algae**	**With algae**	**Without algae**	**With algae**	**Without algae**	**With algae**	**Without algae**	**With algae**
				Smaller particles					
Day 0 (test start)	(–20.3)[1]	–23.6	–23.6	–23.4	–24.3	–23.2	–23.6	–23.8	–23.8
Day 3 (test end)	–33.3	–24.0	–35.9	–25.1	–25.9	–23.5	–25.1	–23.7	–23.9
				Larger particles					
Day 0 (test start)	(–20.3)[1]	–26.2	–26.6	–25.8	–26.1	–26.0	–26.4	–25.3	–25.7
Day 3 (test end)	–33.3	–25.1	–32.3	–21.8	–24.9	–25.2	–25.9	–24.4	–27.2

[1] Initial concentration was too low to provide a representative result.

area of the dispersions while the test vessels were shaking (see Figs. 5 and 6). Lyon et al. (2006) investigated fullerene water dispersions and found that the mean diameter of agglomerates ranged from 2–147 nm, with fractions containing smaller agglomerates having the greatest antibacterial activity. However, toxicity did not increase in proportion to surface area.

4.1.2 Determination of toxicity

Although we used insoluble metal oxides, our experiments demonstrated that toxicity increases with concentration (see Figs. 3, 4, 7, 8). This suggests that inhibition is induced by a particulate and potentially photocatalytic – the spectrum included relevant wave lengths – phenomenon. Both types of TiO_2 particles failed to inhibit algae cells 100 %, with the small particle size achieving a maximum 40 % inhibition of growth rate and 70 % inhibition of yield. Higher concentrations did not increase toxicity. This fact supports the finding that inhibition does not reflect the turbidity of the dispersion (Hund-Rinke and Simon 2006; Aruoja et al. 2009). Previously reported EC_{50} values for algae exposed to dispersed TiO_2 nanomaterials fell within the range 6–44 mg/L (Hund-Rinke and Simon 2006; Wang et al. 2008; Warheit et al. 2007; Aruoja et al. 2009), which is comparable to our results.

Incubation was performed in microtiter plates (200 µl) and Erlenmeyer flasks (100 mL), with the latter showing less variation in calculated EC_x-values. This indicates that microtiter plates are more suitable for experiments with many variables that need to be tested simultaneously to compare the results, whereas Erlenmeyer flasks are more suitable for accurate determination of effects and for experiments that need aliquots to be withdrawn for analysis.

The profile of toxicity over time differed between the larger and smaller nanoparticles, with the smaller particles becoming more toxic over time but the larger particles less so. The basis of this phenomenon is unclear, although it may reflect the more rapid sedimentation of larger particles. This would reduce the effective concentration of the particles by limiting their interactions with the algae, allowing them to recover. The zeta potential of both types of particles was similar and remained constant during the test, so this does not provide an explanation for the differential toxicity.

4.1.3 Surface areas of nanoparticle powders and dispersions

The primary size of the larger particles was greater than the primary size of the smaller particles, and as expected the BET surface area of the smaller particles was greater than that of the larger particles. However, in the aqueous medium, we showed that the specific surface area of the larger particles was greater than that of the smaller particles. This apparent contradiction is explained by the material-specific nature of the measurements. The BET value refers to the powder, and includes the roughness of the surface. In contrast, the specific surface area in the dispersion is measured by dynamic light scattering and represents the hydrodynamic surface.

4.2 Testing filtrates prepared from dispersions

Algal growth was strongly inhibited by filtrates prepared from dispersions containing the highest concentration of nanoparticles (see Tables 3 and 4) indicating that the toxic fraction of the dispersion is represented by components small enough to pass through a 0.22-µm filter. This phenomenon was also noted by Lovern and Klaper (2006) who tested the effect of filtered and unfiltered TiO_2 samples on daphnids. They show the presence of a higher number of small particles in the filtered test medium. As the impurities of the tested nanoparticles due to heavy metals are below the threshold values fixed by the EFSA, it is obvious that the main impurity is water. Therefore, it is concluded that the toxicity of the filtrate is caused by nanoparticles and not by impurities. This assumption is supported by the fact that stirring for 7 d resulted in no toxicity. It is expected that larger agglomerates are less bioavailable.

It is interesting to note that whereas the diluted filtrate had little toxicity, diluted unfiltered dispersions were toxic. This apparent contradiction reflects differences in the way samples below the highest test concentration were prepared. Each TiO_2 dispersion was prepared separately from powder, and the shape of the saturation curve (see Figs. 3 and 4) suggests there was a surplus of the smaller nanoparticles responsible for toxicity. In contrast, the diluted filtrates were prepared by serially diluting the sample with the highest test concentration, resulting in a concomitant reduction in the concentration of smaller, more toxic nanoparticles. It is also possible that further agglomeration of particles in the dispersion samples may have contributed to the discrepancy.

4.3 Method of medium preparation and testing – dispersion versus filtrates

As discussed above, stirring for 7 days appears to eliminate TiO_2 toxicity, a phenomenon that was also observed by Oberdörster et al. (2006) for fullerenes while attempting to simulate environmentally relevant conditions. They found that long-duration stirring resulted in lower toxicity than sonication and solubilization. It is unclear whether long-term stirring simulates environmentally relevant conditions, particularly when compared to the impact of other parameters such as the ionic strength and pH of the medium, both of which significantly influence the formation of agglomerates (Fang et al. 2009). Furthermore, dissolved organic matter may also accelerate agglomeration, as observed for several metal oxide nanoparticles following the addition of

natural pond water (Velzeboer et al. 2008). The addition of fulvic acid to TiO_2 nanoparticles dispersed in a well-defined mineral medium stabilized the agglomerates under conditions that recreated environmentally relevant pH and ionic strength (Domingos et al. 2009).

Whereas the toxicity of dispersions can only be expressed on the basis of nominal concentrations, the toxicity of filtrates can be expressed in terms of either nominal or analytical concentrations. Therefore, the toxicity results for the small TiO_2 particles varied from no toxicity at all, to EC_{20} values of several mg/L (dispersions or filtrates expressed as nominal concentrations) to EC_{20} values of several µg/L (filtrates expressed as analytical concentrations). Effect concentrations are used to trigger hazard statements, and according to Directive 67/548/EEC, not readily biodegradable substances with a low solubility in water (≤1 mg/L) and a log $K_{ow} \leq 3$ are classified as harmful for the aquatic environment if the E_rC/LC_{50} is between 10 and 100 mg/L. If the E_rC/LC_{50} is below 1 mg/L they are classified as very toxic to aquatic organisms. Therefore, the conclusions drawn with respect to the toxicity of nanomaterials and the resulting consequences will differ depending on whether total concentrations from dispersion testing or concentrations in filtrates are used to calculate toxicity indicators. Because information on the toxic fraction of dispersed nanomaterials is still limited, nominal concentrations currently appear to be more suitable for effect characterization. This may be revised, and recalculation of the toxicity may be possible, when more information about the toxic fractions of nanomaterials becomes available. We recommend that short-term dispersion procedures are used to characterize the aquatic ecotoxicity of nanomaterials, since long-term stirring appears unsuitable and inappropriate for this purpose.

4.4 Expression of toxicity – surface versus concentration

Many nanomaterials are functionalized and this affects their behavior (Nowack and Bucheli 2007). Data obtained by Lin et al. (2006) for TiO_2 clearly indicate the importance of the specific surface area in controlling photocatalytic reactivity. Therefore, specific surface area is proposed as a potentially more suitable indicator for nanomaterial toxicity than concentration. Under our experimental conditions, the specific surface area, based on the hydrodynamic diameter, changed during the incubation period (see Figs. 5 and 6) and it is unclear whether the toxicity of nanomaterials primarily reflects their specific surface area at the beginning of a test, at the end-point, or a mean value. Furthermore, our data concerning the toxicity of filtrates (see Tables 3 and 4) indicate that only a fraction of the agglomerates, i. e. those <0.22 µm in diameter, are responsible for the observed toxicity. Although the TiO_2 concentration was reduced to ~100 µg/L, significant toxicity was still observed. Considering these results, there appears to be no advantage in using the hydrodynamic surface area rather than the concentration to define toxicity. While the hydrodynamic surface presumably underestimates the relevant surface area, the BET value as an indicator for the surface of the dry TiO_2 nanoparticles gives an overestimation. The d(0.10) value is also unsuitable. This is the maximum diameter of the smallest 10 % of agglomerates in the dispersion, but this must still be too high a threshold to meaningfully represent the toxic component of the dispersion. The d(0.10) value is inversely related to specific surface area, but neither of these parameters appear to have a simple relationship with toxicity (see Table 2). We therefore recommend that concentration remains the principal measurement used to characterize the toxicity of nanoparticle dispersions until the toxic fraction has been investigated in more detail.

5 Conclusions

- The toxicity of TiO_2 nanoparticle dispersions can depend on the preparation method (see Table 2). Long-term stirring seems to reduce toxicity. In case the OECD guideline concerning algae toxicity is applied without further amendment of the test medium, a combination of short-term stirring and ultrasonication is recommended as a convenient approach to assess the ecotoxicity of such preparations, for example 1 min stirring to distribute the material homogenously in the vessel followed by 3 min ultrasonication to disperse agglomerates.
- Filtration, as proposed in the OECD test guidance no. 23 for the testing of difficult substances, is not yet recommended for TiO_2 nanoparticles. The toxic fraction of the nanoparticles and their agglomerates is still poorly characterized. The most suitable filtration procedure is not yet known and results might therefore vary if different filter types and pore sizes are used, since this might result in the retention of relevant toxic components.
- The surface area of functionalized nanomaterials may have more of an influence on their ecotoxicity than the absolute concentration. Nevertheless, there appears to be no advantage in using the total hydrodynamic surface instead of concentration to study ecotoxicity. This is because only a small fraction of the nanomaterial is responsible for toxicity, and that component is not yet fully understood. Furthermore, changes in surface area during the incubation period due to agglomeration of TiO_2 (see Figs. 5 and 6) may interfere with the interpretation of any relationship between surface area and toxicity. Therefore, concentration remains the most appropriate parameter for the investigation of TiO_2 ecotoxicity. This may be revised, and recalculation of the toxicity may be possible, when more information about the toxic fractions of nanomaterials becomes available.

References

Adams LK, Lyon DY, Alvarez PJJ (2006) Comparative eco-toxicity of nanoscale TiO_2, SiO_2 and ZnO water dispersions. Water Res 40:3527–3532

Aruoja V, Dubourguier H-C, Kasemetsa K, Kahru K (2009) Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Sci Total Environ 407:1461–1468

Beck-Speier I, Dayal N, Karg E, Maier KL, Roth C, Ziesenis A, Heyder J (2001) Agglomeration of ultrafine particles of elemental carbon and TiO_2 induce generation of lipid mediators in alveolar macrophages. Environ Health Persp 109:613–618

Choi Y, Umebayashi T, Yoshikawa M (2004) Fabrication characterization of C-doped anatase TiO_2 photocatalysts. J Mat Sci 39:1837–1839

DIN 32645 (1994) Nachweis-, Erfassungs- und Bestimmungsgrenze – Ermittlung unter Wiederholbedingungen (Begriffe, Verfahren, Auswertung). Beuth Verlag, Berlin

DIN 38406-29 (1999) Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung; Kationen (Gruppe E); Teil 29: Bestimmung von 61 Elementen durch Massenspektrometrie mit induktiv gekoppelten Plasma (ICP-MS). Beuth Verlag, Berlin

Domingos RF, Tufenkji N, Wilkinson KJ (2009) Aggregation of titanium dioxide nanoparticles: role of a fulvic acid. Environ Sci Technol 43:1282–1286

Fang J, Shan X, Lin J, Owens G (2009) Stability of titania nanoparticles in soil dispersions and transport in saturated homogenous soil columns. Environ Pollut 157:1101–1109

Finney DJ (1984) Statistical Method in Biological Assay, 2nd edn. London

ToxRat® Professional 2.10. ToxRat® Solutions, Alsdorf

Hund-Rinke K, Simon M (2006) Ecotoxic effects of photocatalytic active nanoparticles (TiO_2) on algae and daphnids. Environ Sci Pollut Res 13:225–232

ISO 14442 (2006) Water quality – guidance for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water

ISO 8692 (2004) Water quality – Freshwater algal growth inhibition test with unicellular green algae

Lin D, Xing B (2007) Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. Environ Pollut 150:243–250

Lin H, Huang CP, Li W, Ni C, Shah SI, Tseng Y-H (2006) Size dependency of nanocrystalline TiO_2 on its optical property and photocatalytic reactivity exemplified by 2-chlorophenol. Appl Catal B 68:1–11

Linkous CA, Carter GJ, Locuson DB, Ouellette AJ, Slattery DK, Smitha LA (2000) Photocatalytic inhibition of algae growth using TiO_2, WO_3 and cocatalyst modifications. Environ Sci Technol 34:4754–4758

Lovern SB, Klaper R (2006) *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. Environ Toxicol Chem 25:1132–1137

Lyon DY, Adams LK, Falkner JC, Alvarez PJJ (2006) Antibacterial activity of fullerene water dispersions: effects of preparation method and particle size. Environ Sci Technol 40:4360–4366

Nowack B, Bucheli TD (2007) Occurrence, behavior and effects of nanoparticles in the environment. Environ Pollut 150:5–22

Oberdörster E, Zhu S, Blickley TM, McClellan-Green P, Haasch ML (2006) Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerenes (C60) on aquatic organisms. Carbon 44:1112–1120

OECD Test Guideline No. 201 (2006) Freshwater algae and cyanobacteria, growth inhibition test

OECD Series on Testing and Assessment/Adopted Guidance and Review Documents – No. 23 (2000) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures

Velzeboer I, Hendriks AJ, Ragas MJ, van den Meent D (2008) Nanomaterials in the Environment – Aquatic Ecotoxicity Tests of some Nanomaterials. Environ Toxicol Chem 27:1942–1947

Wang J, Zhang X, Chen Y, Sommerfeld M, Hu Q (2008) Toxicity assessment of manufactured nanomaterials using the unicellular green alga *Chlamydomonas reinhardtii*. Chemosphere 73:1121–1128

Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM (2007) Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. Toxicol Letters 171:99–110