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The challenges of testing metal and metal oxide nanoparticles in algal bioassays: titanium dioxide and gold nanoparticles as case studies

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Abstract

Aquatic toxicology of engineered nanoparticles is challenged by methodological difficulties stemming partly from highly dynamic and poorly understood behavior of nanoparticles in biological test systems. In this paper scientific and technical challenges of testing not readily soluble nanoparticles in standardised algal growth inhibition tests are highlighted with specific focus on biomass quantification methods. This is illustrated through tests with TiO_2 and Au nanoparticles, for which cell-nanoparticle interactions and behavior was studied during incubation. Au NP coating layers changed over time and TiO_2 nanoparticle aggregation/agglomeration increased as a function of concentration. Three biomass surrogate measuring techniques were evaluated (coulter counting, cell counting in haemocytometer, and fluorescence of pigment extracts) and out of these the fluorometric methods was found to be most suitable. Background correction was identified as a key issue for biomass quantification, complicated by algae-particle interactions and nanoparticle transformation. Optimisation of the method is needed to reduce further particle interference on measurements.

Keywords: TiO_2, Au, growth inhibition, ecotoxicity, guidelines

Introduction

The ability to test for the hazard potential of inorganic engineered nanoparticles is a fundamental prerequisite for their risk assessment. A number of international guidelines and standardized methods are available for testing of base-set organisms (fish, crustaceans, and algae) used for aquatic hazard and risk assessments (ECHA 2008). Employing the accumulated knowledge related to the use of these standardized tests, with fully defined synthetic media compositions, has also been suggested as a way of systematically increasing the scientific insight into environmental fate and effects of nanoparticles (Baun *et al.* 2008). Most of the aquatic test guidelines, such as OECD guideline 201 (OECD 2006) and ISO 8692:2004 (ISO 2004) for algal growth inhibition tests, requires that the tested chemicals are water soluble. Typically this will not apply to nanoparticles, which are often partly soluble, slightly soluble or insoluble (i.e., not readily soluble). Hence, they generally form suspensions, with varying degree of aggregation/agglomeration and stability, rather than dissolve in water. This raises a fundamental concern whether available standard test methods are applicable to nanoparticles as it is at present uncertain how to perform tests in order to obtain the most meaningful results (e.g., Hartmann *et al.* 2010; Tiede *et al.* 2009).

In a review of the applicability of existing OECD ecotoxicity test guidelines to nanomaterials by the OECD Working Party on Manufactured Nanomaterials (WPMN), a number of specific shortcomings were identified mainly related to characterization, exposure preparation, quantification and monitoring concentrations, and dose-metrics (OECD 2009). It was suggested that documents, containing guidance on nano-specific test concerns, might be a better option rather than extensive modifications of all OECD ecotoxicity test guidelines (OECD 2009). It is thus acknowledged that guidance is needed for testing of nanomaterials. However, the use of existing test guidelines for regulatory testing purposes has been recommended until such nano-specific test guidelines are available (European Commission 2008). While careful description of test systems and thorough particle characterization has been advocated as short-term measure, allowing for future evaluation and analysis of observed (eco)toxicological effects of engineered nanoparticles (Tiede *et al.* 2009), the present aim must be to gain a deeper fundamental understanding of nanoparticle behavior in test systems, their interactions with test organisms and influence on end-point measurement methods. On basis of this, guidance on appropriate testing strategies can be developed.

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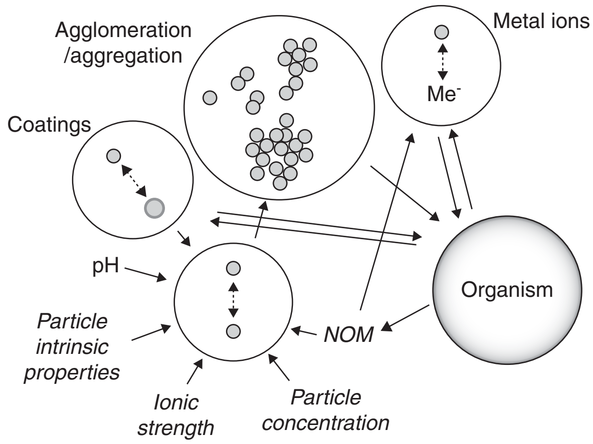

Figure 1. Dynamic two-way interactions between organisms and nanoparticles in a dynamic test system and influence of environmental factors.

In many ways algal tests are different from other aquatic ecotoxicological tests (OECD 2000) which is related to the testing of cell cultures rather than e.g., invertebrates, vertebrates or larger plants. Handling of difficult substances in algal tests therefore also represents specific challenges. Large variability in EC_{50} values for nanoparticle effects on algal growth is reported (Menard *et al.* 2011), which further highlights the importance of test reliability and reproducibility and of interpretability of test outcomes. The applicability of existing algal test guidelines is impaired by the fundamental differences between nanoparticles and water soluble chemicals. This includes the dynamic nature of the nanoparticles in aqueous media resulting from e.g., aggregation/agglomeration and sedimentation (Keller *et al.* 2010). This may be increased due to algal exudates (Koukal *et al.* 2007), and biomodifications of nanoparticle properties (Roberts *et al.* 2007) as illustrated in Figure 1 – all of which impairs the quantification and controlling of actual exposure conditions. Hence, a number of environmental factors are known to influence nanoparticle behavior including pH, ionic strength and particle concentration (e.g., Bai *et al.* 2010; von der Kammer *et al.* 2010; Tiede *et al.* 2009). The presence of organisms also itself influences nanoparticle behavior (and thereby exposure) both directly by removing coating layers and indirectly by changing some of these controlling factors. The two-way interactions between organisms and nanoparticles in aqueous suspensions are illustrated in Figure 1.

Additional factors impairing test result interpretations are time- and concentration-dependent aggregation/agglomeration in algal growth tests as pointed out by Hartmann *et al.* (2010) and Hund-Rinke *et al.* (2010). Also affinity of algae cells to nanoparticles may influence the physical appearance of algal cells in the test system, e.g., causing formation of cell-particle aggregates. There is evidence of nano-specific effect mechanisms which involve close interactions between nanoparticles and cell surfaces (see e.g., Rogers et al. 2010). Cell encapsulation as a direct or indirect cause of decreased growth has also been suggested by for example Schwab *et al.* (2011), Hartmann *et al.* (2010), Aruoja et al. (2009), van Hoecke *et al.* (2009), and Hund-Rinke & Simon (2006). To minimize shading effects, and hence variability in the test results, it has been suggested by Handy *et al.* (2012a) and Handy *et al.* (2012b) that algal test protocols could be amended for nanoparticles with respect to light intensity, which has previously been highlighted as an important issue for algal tests with colored substances (Cleuvers & Ratte 2002). Both the dynamic nature of the test systems and the affinity of algal cells to nanoparticles represent major challenges causing difficulties in (1) describing exposure and (2) distinguishing the algal phase from nanoparticles hampering the use of some biomass (surrogate) quantification techniques. As also pointed out recently by Handy et al. (2012a), nanoparticle interference with algae growth quantification techniques is a potential source of error. The most common methods used for quantification of biomass are based on cell counting (traditional microscopy or automated counting) or fluorescence measurements of extracted pigments. When dispersions are tested in algal growth inhibition tests high background particle numbers are known to disturb biomass measurements – especially when using particle counters or spectrophotometric methods. For this reason background corrections (test suspensions without algae) are recommended (ISO 2006).

Testing is further complicated by the fact that different types of nanoparticles present different challenges due to their diverse nature and behavior in aquatic test systems. For instance, metal nanoparticles, such as gold nanoparticles, are often coated to form stable colloidal dispersions (Daniel & Astruc 2004), and metal oxide nanoparticles, such as TiO_2 nanoparticles, may form aggregates/agglomerates almost immediately in aqueous media (Keller et al. 2010). Aggregation/agglomeration is dominated by factors such as ionic strength and the presence of natural organic matter (Figure 1) (Keller *et al.* 2010). Due to their widely different behavior Au nanoparticle colloidal dispersions and aggregating TiO_2 nanoparticles thereby represent two very different cases in nanoecotoxicology.

The aim of this article is, through a series of tests with TiO_2 and Au nanoparticles, to investigate scientific and technical challenges of testing not readily soluble nanoparticles in standard algal tests related to determination and interpretation of effects on algal growth rates. Methods for biomass quantification (including background correction methods) are evaluated and observed effects are discussed in relation to visual and analytical observations of the algal test systems during the growth period. As an outcome, new recommendations for improved reliability and interpretability of results obtained in algal growth inhibition tests with nanoparticles are presented.

Materials and methods

Nanoparticles—sources, synthesis and preparation of test suspensions

Au nanoparticles used in the present work were synthesized in a buffered glucose-starch solution based on a modification of a recipe from Engelbrekt *et al.* (2009). Glucose and starch are reducing and protecting agents, respectively. Different sizes of Au nanoparticles can be obtained in 2-(*N*-morpholino)

Table I. Suspensions of nanoparticles tested for stability by measuring absorbance over time.

Sample no.	Stock suspension preparation	Test suspension preparation	Final pH
1	250 mg/L Au diluted to 60 mg/L in algal medium and pH adjusted to 7.3	Diluted to 10 mg/L in algal medium	7.5
2	1 g/L TiO_2 P25 in MilliQ water	Diluted to 10 mg/L in algal medium	7.6
3	1 g/L TiO_2 P25 in algal medium.	Diluted to 10 mg/L in algal medium	7.6
4		Diluted to 40 mg/L in algal medium	7.6
5		Diluted to 100 mg/L in algal medium	7.6
6	–	10 mg/L TiO_2 in algal medium suspended directly in media as dry powder	7.6
7	1 g/L TiO_2 P25 in algal medium.	Diluted to 10 mg/L in MilliQ water	n.q.
8		Diluted to 100 mg/L in MilliQ water	n.q.

n.q., not quantified due to issues of measuring pH in MilliQ water.

ethanesulfonic acid (MES) and phosphate buffer. For this study $HAuCl_4$/glucose/starch/MES solution was heated to 90°C for 1 h with stirring during synthesis. The emerging colloidal dispersion was strongly red colored with a final pH around 6. The Au concentration in the dispersion at the time of testing was determined by 48 h metal extraction in aqua regia in a 1:1 volume ratio and diluted for analysis by inductively coupled plasma optical emission spectrometry (Optima 5300DV, Perkin Elmer) with ICP multi-element standard (CertiPUR, Merck). The stock concentrations of the colloidal dispersion used for testing was 250 mg/L Au. Prior to testing the dispersion was diluted 60 mg/L Au in concentrated algae test medium and pH adjusted to 7.3 by 1 M NaOH. Algal test medium was prepared according to OECD Test Guideline 201 (OECD 2006). Test suspensions were prepared by diluting this suspension to test concentrations of 1.9–30 mg/L. In the same way a solution containing only glucose/starch/MES in concentrations identical to that of the Au nanoparticle dispersions (10 mM MES adjusted to pH 7 with KOH, 0.6 wt% soluble starch and 10 mM glucose) was prepared (henceforth termed "Starch Control") to investigate its influence on both algal growth and biomass quantification techniques.

AEROXIDE® TiO_2 P25 nanoparticles (nominal primary particle diameter: 21 nm) were procured from Evonik Degussa. TiO_2 stock suspensions were prepared by suspending TiO_2 particles in algal test medium in a concentration of 1 g/L followed by 10 min sonication in a water bath (Model 3510, Branson). Test suspensions were prepared by diluting this suspension to test concentrations of 35–560 mg/L. The stock suspension was kept at 5°C in the dark and sonicated again for 10 min prior to preparation of test suspensions.

Stability of nanoparticle suspensions

The stability of suspensions of the tested nanoparticles was tested by placing 10 mg/L suspensions in algal media in quartz cuvettes and measuring the light absorbance during a period of 6–10 h at 338 nm (TiO_2) or 523 nm (Au) (Cary Bio50 UV-VIS spectrophotometer). Additional suspensions and treatments of TiO_2 nanoparticles were prepared for spectroscopy by adding dry powder TiO_2 nanoparticles to a 500 mL measuring flask and filling the flask with the relevant media. This was followed by vigorous shaking of the flask for 30 s and sonication in a water bath for 10 min (Model 3510, Branson). An overview of the tested suspensions is given in Table I.

Transmission electron microscopy and particle characterization

The particle core size was characterized by BF-TEM (Tecnai T20 G2 TEM, FEI Oregon, USA, operated at 200 kV). Copper grids with holey carbon support films from Agar Scientific (Stansted, UK) were fixed in vertically mounted tweezers. One drop of Au nanoparticle suspension was placed on the grid and the majority sucked away from the side with tissue paper or pipette. The grids were covered to protect from dustfall and dried in air overnight.

The hydrodynamic diameter and size distributions of suspended particles were determined by means of nanoparticle tracking analysis using Nanosight LM10, NTA 2.1 software (NanoSight Ltd., Wiltshire, UK). The measured suspensions were diluted to appropriate concentrations in algal medium and injected into the laser block followed by adjustment of lateral position and focus. A quick flush with additional solution was applied and the system was allowed to stabilize for 30 s prior to recording to ensure reproducibility. Zeta potential and hydrodynamic diameter (by dynamic light scattering) were measured using a Zetasizer Nano ZS (Malvern Instruments, UK).

Algal growth rate inhibition tests

The algal growth inhibition test procedure was performed according to OECD Test Guideline 201 (OECD 2006) with green algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz) as test species. The OECD algal medium was used to prepare a dilution series of the test substances. All concentrations and controls were inoculated with an algal culture in exponential growth phase to a density of 5×10^4 cells/mL. The increased cell density, compared to the 10^4

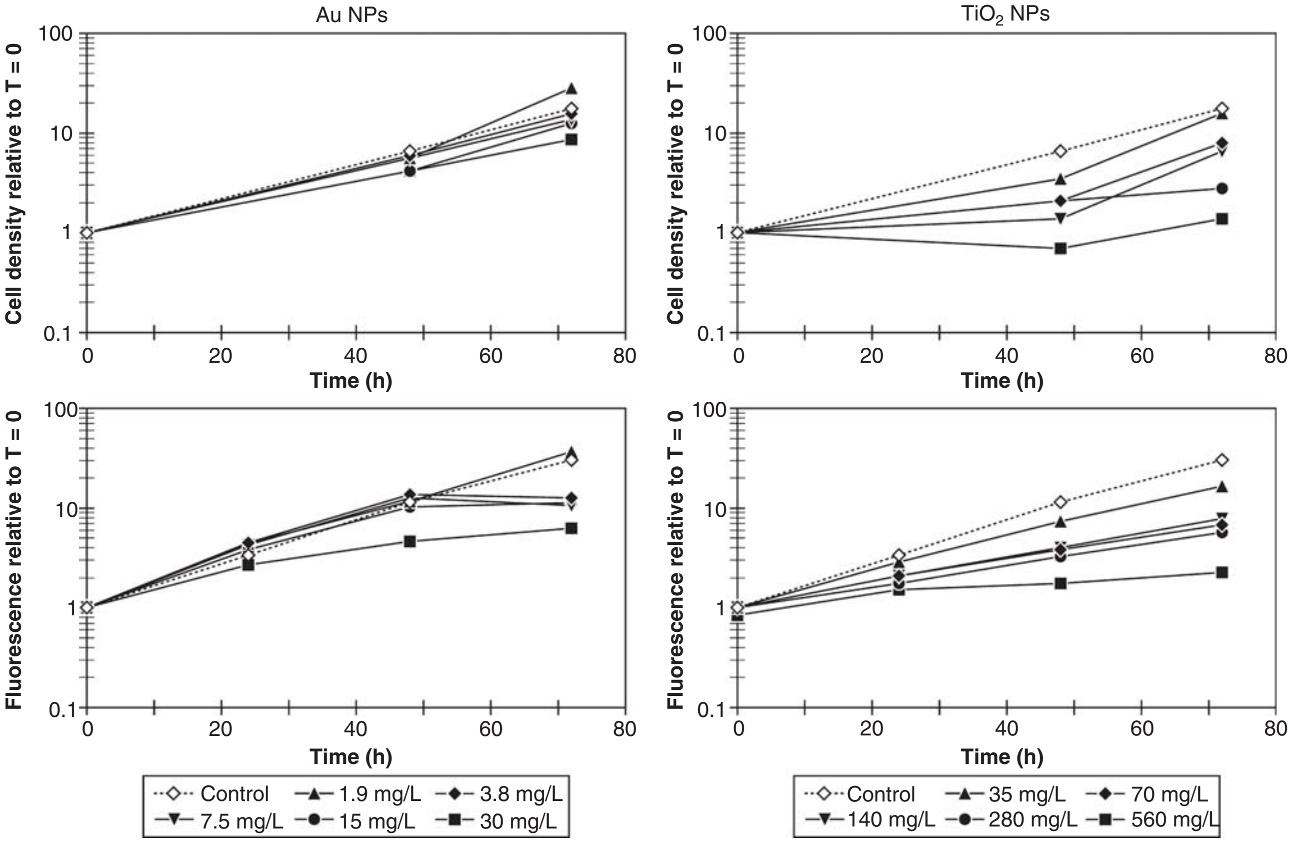

Figure 2. Growth curves obtained by two different ways of biomass quantification (cell counting in haemocytometer and fluorescence of extracted pigments) in algal cell cultures (*P. subcapitata*) exposed to Au or TiO_2 nanoparticle suspensions. Growth rates were determined by linear regression based on all sampling times. The average control growth rate was 1.1 d^{-1} for the control replicates based on fluorescence of acetone pigment extraction method. Based on haemocytometer counts it was found to be 0.9 d^{-1}. The difference may be explained by inaccurate determination of initial cell density. The coefficient of variance for 72 h growth rates of control culture was 8%. In exposed cultures the coefficient of variance ranged from 1–12% (with a general trend of higher variance for higher concentrations) for Au exposed cultures. For TiO_2 exposed cultures there was no clear trend. The coefficients of variance were generally 11–17% but for one exposure concentration the coefficient of variance was 54%. The higher coefficients of variation for exposed cultures are likely to be caused by particle interference. For cell counting in haemocytometer the mean coefficient of variation was 6% between control replicate growth rates and varied from 2–17% for the cultures exposed to Au nanoparticles. For TiO_2 exposed cultures coefficients of variation cannot be calculated due to the test design (see *Determination and characterization of algal biomass*).

cells/mL recommended by OECD 201 (OECD 2006), was chosen to increase the signal/noise ratio in the biomass determinations, to differentiate algal pigment fluorescence from background noise resulting from particulate matter and color of the test compounds. This modification was found to be acceptable as the OECD validity criteria for 72 h growth rate (min.0.92 d^{-1}) was fulfilled (average control growth rate: 1.1 d^{-1} based on fluorescence measurements of acetone extracted pigments, see Figure 2). All test concentrations were tested in triplicates with ten controls, containing only test medium and algae. Five concentrations of TiO_2 (35–560 mg/L, nominal concentrations) and Au (1.9–30 mg/L, measured initial concentrations) nanoparticles were tested. The "Starch Control" solution was used as a reference and tested in concentrations corresponding to nominal Au nanoparticle concentrations of 1.9–15 mg/L (46–365 mg/L starch) to determine the effects of the starch, glucose and MES in the Au nanoparticle dispersions. A mini-scale test was applied in this study (Arensberg *et al.* 1995). All test glass vials (20 mL) with algae and test solutions (4 mL) were incubated for 72 h. Vials were closed with lids with a small hole to allow for CO_2 diffusion. The containers were placed on a shaker (200 rpm) at 20 ± 2°C and continuously illuminated at 86–109 μE/m^2/s (measured under the test vessel). The light source was a cold light fluorescent tube emitting light in the visible spectrum (Phillips TL-D 30W/33-640 SLV). Light intensity in the test setup was measured using a LI-COR light meter (model LI-189) with an attached quantum sensor, measuring light in the wavelength range 400–700 nm. The tests were conducted at a pH of 7.3–7.9. pH did not change more than 0.1–0.2 units for the individual samples during the 72 h exposure period.

Determination and characterization of algal biomass

The algal biomass was quantified using three different techniques:

(1) Cell counting by use of a Coulter® Counter (Multisizer™ Z2, Beckman Coulter) attached to a computer with COULTER®AccuComp® version 3.01 software (Beckman Coulter Corporation 2000). The size range was set to 2–6.5 μm based on the algae cell size. Particle number and size distributions were recorded and the result expressed as particle number per mL.

Table II. Characterisation of Au and TiO_2 nanoparticles in OECD algal test medium. TEM, DLS and Zeta potential analysis was performed for samples with comparable test concentrations of 30 mg/L Au and 35 mg/L TiO_2 respectively.

	Primary particle diameter [nm]		Effective particle diameter [nm]		Zeta potential [nm]
Method	Nominal	TEM	DLS	NTA	
Au	N/A	25 ± 4	51 ± 8	46 ± 22	–20 ± 20
TiO_2	21[a]	23 ± 7	1120 ± 50	n.q.	–14 ± 3

[a]Degussa Evonic (2010); N/A, Not applicable (in-house synthesis); n.q., Not quantifiable (due to technical limitations).

The initial cell density of the stock algae culture was measured prior to test inoculation. Due to a limited test volume, samples of 1 mL were taken for Coulter Counting only at 72 h and diluted in isotonic water to a final volume of 10 mL.

(2) Counting of cells was done in a haemocytometer (Thoma, 0.1 mm depth) using an optical microscope (Olympus BH-2) with phase contrast. The cell density in a 200 μL sample was determined after 48 and 72 h of incubation. In the preliminary test only one replicate from each test concentration was counted after 72 h incubation. In the final test the cell density of all control replicates and replicates exposed to Au nanoparticles was determined after 72 h. Mixed samples of the three replicates were counted after 48 h for cultures exposed to Au nanoparticle and after 48 and 72 h for cultures exposed to TiO_2 nanoparticle. It should be noted that particle-cell aggregation/agglomeration made counting of algae exposed to TiO_2 nanoparticles difficult and time consuming.

(3) Fluorescence of acetone extractions of algal pigment, as described by Mayer *et al.* (1997) was used as a biomass measure. The fluorescence of the samples were measured on a fluorescence spectrophotometer (Hitachi F-2000) using an excitation wavelength of 430 ± 5 nm and emission wavelength of 671 ± 20 nm. Samples of 0.4 mL were taken and extracted at times 0, 24, 48 and 72 h. Extraction time was 48 h (as will be described further in ***Fluorescence of extracted pigments***.)

Initial biomass quantification

The cell density of the algal inoculum culture was determined by Coulter Counting or by counting in haemocytometer, respectively. Due to low sensitivity of both these methods at low cell density, initial cell densities in the tests were calculated based on inoculum cell density values. This procedure is prescribed by OECD as it provides the greatest precision unless a method with high precision at low cell densities (such as flow cytometry) is used to determine inoculated biomass concentration (OECD 2006).

The initial fluorescence of extracted algal pigment was overshadowed by nanoparticle interference for the higher concentrations of nanoparticles. Hence, the average control culture fluorescence (no nanoparticles added) was used as the initial fluorescence value for all test concentrations.

Statistical analysis of algal test results

Concentration-response curves were estimated based on growth rates by use of a nonlinear-regression program (Christensen *et al.* 2009) assuming log-normal distribution. EC-values were determined from the concentration-response curve with corresponding 95% confidence limits.

Results

Particle characteristics and test system dynamics

Particle characterization in algal test medium

Characteristics of the two nanoparticles in suspension were determined as described in *Transmission electron microscopy and particle characterization* and the results can be seen in Table II.

The average hydrodynamic diameter of the Au nanoparticles in algal medium was found to be 51 ± 8 nm by dynamic light scattering (DLS) measurements. This was confirmed by NTA measurements, where an average size of 46 ± 22 nm was observed, with a well-defined population of particles with a hydrodynamic diameter around 40 nm. For TiO_2 nanoparticles it was not possible to measure the particle diameter by NTA due to the irregular shape and large size of the TiO_2 aggregates, which caused irregular light scattering. However, DLS results show that, under these experimental conditions, TiO_2 nanoparticles particles form large (micron-sized) agglomerates/aggregates.

Particle suspension stability

The stability of TiO_2 and Au nanoparticle test suspensions (Table I) was monitored over time by UV-VIS spectroscopy (Figure 3). The results show that Au nanoparticles suspended in OECD algal test medium (Figure 3, sample 1) were very stable during the measuring period of 360 min. The rate of sedimentation of TiO_2 nanoparticles in algal medium increased with increasing concentrations (10, 40, and 100 mg/L TiO_2) (samples 3, 4 and 5). The preparation method of the test suspension had only a minor effect on the sedimentation rate. Visual inspection of the 1 g/L TiO_2 stock

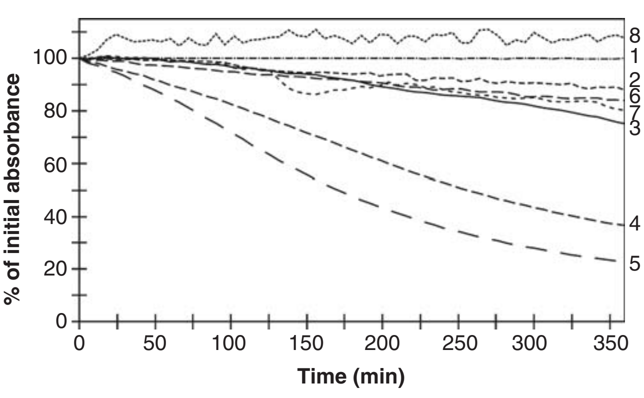

Figure 3. Reduction of absorbance (λ = 338 nm (TiO_2) and λ = 523 nm (Au)) as a result of sedimentation of Au and TiO_2 nanoparticle suspended in different media and concentrations. Sample 1 is absorbance of a colloidal dispersion of Au nanoparticles diluted to 10 mg/L in algal medium. Samples 7 and 8 correspond to 10 and 100 mg/L TiO_2, respectively, suspended in MilliQ water. Samples 2, 3, 4, 5 and 6 are 10, 10, 40, 100 and 10 mg/L TiO_2, respectively, suspended in OECD test medium.

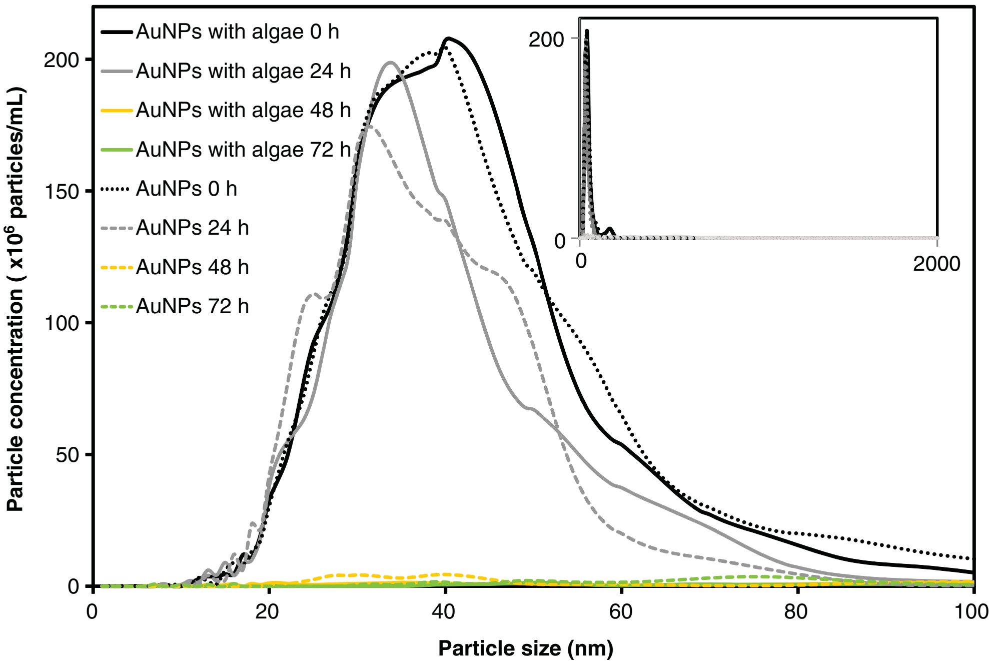

Figure 4. Development in Au nanoparticle hydrodynamic diameter distributions over a 72 h incubation period in an algal growth inhibition test measured by NTA. The inserted graph shows size distribution in the range from 0 to 2 μm. The samples (1.9 mg/L) were incubated on a shaking table at 20 ± 1°C and continuously illuminated at 86–109 E/m^2/s. Samples only containing Au nanoparticles were incubated along with algal cultures exposed to the nanoparticles.

suspension in MilliQ water showed high stability compared to the equivalent stock suspension in algal medium. In MilliQ water the 100 mg/L TiO_2 suspension (sample 8) was stable with some fluctuations in absorbance and negligible sedimentation, while the 10 mg/L TiO_2 dilution (sample 7) showed sedimentation rates similar to suspensions prepared in algal medium. This could be related to the negative surface charges and surface-confined counter ions (including protons) of the TiO_2 nanoparticles, in turn leading to attractive forces between particles through divalent cation bridges. Such effects would be expected to be more predominant for more diluted samples and hence increasing stability is expected with increasing TiO_2 nanoparticle concentrations. Similarly these effects can also be stimulated by Zn^{2+} and transition metals such as Fe^{3+} in the growth medium, which could contribute to explaining the different behavior in algal test media compared to MilliQ water.

These results show that differences in exposure in a standard concentration-response setup may not only be due to the intended differences in test concentrations: different concentrations within the same test particle may differ in behavior and aggregation/agglomeration. This in turn can be assumed to influence bioavailability and particle-cell interactions.

Particle size distributions in the presence of algae

The initial characteristics of the Au nanoparticle dispersions determined by NTA and DLS can be seen in Table II and from Figure 4. A similar pattern was found in the presence of algae (Figure 4). A slight reduction in average hydrodynamic diameter occurred at 24 h and finally the Au nanoparticle population at 40 nm was not observed at 48 h, neither with nor without algae present. A possible reason could be that the coating layer was degraded in the medium causing the particle size to decrease after 24 h. After additional removing of coating material, Au nanoparticles start to aggregate and conglomerate. This can explain the drastic decrease after 48 h, where a very low concentration of particles could be detected below 2 μm. This indicated either formation of larger sized aggregates or adsorption onto a growing biomass consisting either of algae or, as algae cultures will almost always contain some bacteria (ISO 2006), other microorganisms thriving in the favourable light and temperature conditions, combined with relatively high starch concentrations.

The irregular shape and large size of the TiO_2 aggregates caused light scattering, which hampered reliable size determinations by NTA (Nanosight LM10). Populations of smaller particle sizes were observed but also agglomerates in the μm size range were detected. Repeated measurements provided inconsistent results. TiO_2 is known to form larger agglomerates/aggregates of several hundred nanometers in algal media (Hartmann *et al.* 2010), which was also confirmed by DLS measurements (agglomerate/aggregate sizes of 1120 ± 50 nm). These are expected to increase in size over time similarly to findings for ZnO nanoparticles (Bai *et al.* 2010).

The primary particle sizes of individual particles were also measured by TEM micrographs at 0, 24, 48, and 72 h. These measurements provide information on core sizes, as opposed to the hydrodynamic diameter reported in *Particle characterization in algal test medium*, which also include the coating layer and diffuse double layer. The diameter of Au nanoparticles remains unchanged both with and without algae present despite the occurrence of agglomerates after 48 h. The measured average diameter at 0, 24, 48 and 72 h is 26 ± 5, 23 ± 5, 25 ± 3 and 24 ± 3 nm,

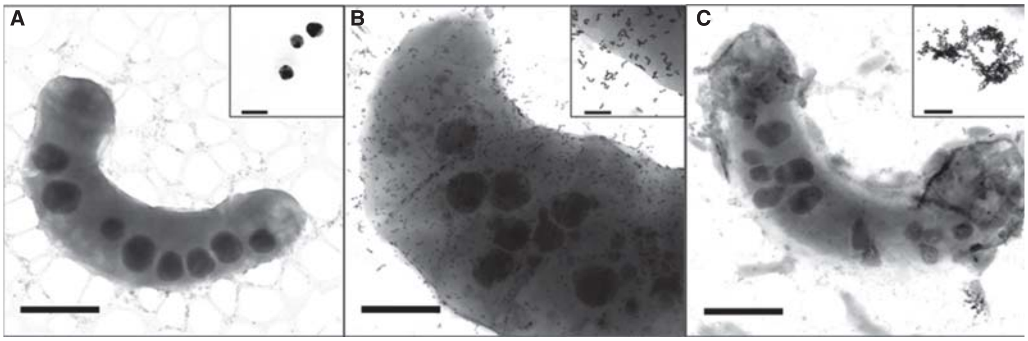

Figure 5. TEM images of A: Algal cell immediately after exposure to 1.9 mg/L Au nanoparticles (scale bar is 2 μm). Insert shows individual Au nanoparticles (scale bar is 40 nm). B: Algal cell with attached Au nanoparticles after 24 h exposure to 3 mg/L (scale bar is 2 μm). Insert shows attached Au nanoparticles at the edge of an algal cell (scale bar is 300 nm). C: Algal cell after 72 h exposure to 1.9 mg/L with no apparent attachment of Au nanoparticles (scale bar is 2 μm). Insert shows aggregate of Au nanoparticles (scale bar is 250 nm).

respectively, without the presence of algae and 25 ± 3, 24 ± 3, 23 ± 2 and 23 ± 3 nm, respectively, with algae present. The constant core size further indicates that changes in hydrodynamic diameter are related to changes in the coating layer thickness.

Interactions with test organisms

The interactions between nanoparticles and algae cells were investigated by TEM micrographs at 0, 24, 48, and 72 h. Little or no interaction between particles and cells were seen for Au nanoparticles when incubation was initiated (Figure 5A). At 24 h increased association of nanoparticles with the cell surface was observed (Figure 5B) and finally at 72 h the appearance of the Au nanoparticle agglomerates on the cell surface changed in shape, seemingly forming more compact structures with distinct shapes.

For algae exposed to TiO_2 nanoparticles a large degree of particle attachment to the cell surfaces was seen throughout the incubation (Figure 6). Some indications of cell deformation was also observed (Figure 6C), though no final conclusions on this can be drawn due to the possible influence of sample preparation (drying) prior to TEM imaging and possible destruction of the sample during measurements. However, comparing TEM images of a non-exposed algae cell to light microscopy images the shape of the cells do not seem to change as a result of TEM sample preparation (see Supporting Information)

Evaluation of techniques for biomass quantification

Quantification of biomass surrogates was attempted by use of cell counting on Coulter Counter and in a haemocytometer as well as by fluorescence measurements of extracted pigments.

Coulter counting

When counting samples containing nanoparticles, but without algae, by Coulter Counting, particles were detected within the measuring size range of 2–6.5 μm for both particle types, showing the presence of much larger agglomerates/aggregates than detected by NTA and TEM (Table II). This may be caused by the dilution in isotonic water, necessary for the counting, leading to the formation of larger agglomerates/aggregates that interfere with the measurements. For TiO_2 background measurements a linear relationship was found between nominal particle concentrations and particle counts. However, the particle number counts for Au nanoparticles reached a maximum and then decreased at the highest concentrations. The subtraction of background values for nanoparticle interference resulted in erroneous determinations of biomass. This resulted from non-linear background particle numbers for Au nanoparticles as accurate determination of the algal densities was complicated by the fact that the combination of algae and nanoparticles results in formation of aggregates of larger sizes (Supporting Information). The formation of algae-nanoparticle aggregates hence changed the size distribution of the samples compared to size distributions of both the pure algae culture and the nanoparticle background samples, respectively (i.e., the presence of algae affected the nanoparticle size distribution and thereby the particle number within the measuring size range and vice versa). Problems resulting from background subtraction of Coulter Counter measurements still had a large influence on the accuracy of the measurements after 72 h for both particle types. This resulted for example in "negative" cell densities for the algal culture exposed to 560 mg/L TiO_2 after subtraction of the background value. Based on these observations the usefulness of cell counting on a Coulter Counter in algal tests with nanoparticles is questionable. The interactions between algal cells and nanoparticles makes these counts a "black box" and a critical evaluation of the obtained results is therefore always needed to avoid misinterpretation of the data. In our case, the problems related to particle background from Coulter Counter values for both particle types hampered the use of these for calculations of growth rate inhibitions.

Haemocytometer

While cell counting in haemocytometer was relatively straight-forward for algal cultures exposed to Au nanoparticles, the counting of TiO_2 samples was hampered by particle aggregation/agglomeration, especially at the higher concentrations. This meant that for all tested concentrations of TiO_2 (>35 mg/L) reliable counting of algae was difficult due to the localization of algal cells among/inside particle aggregates. This method is therefore not recommended for highly aggregating nanoparticles, especially not if they are

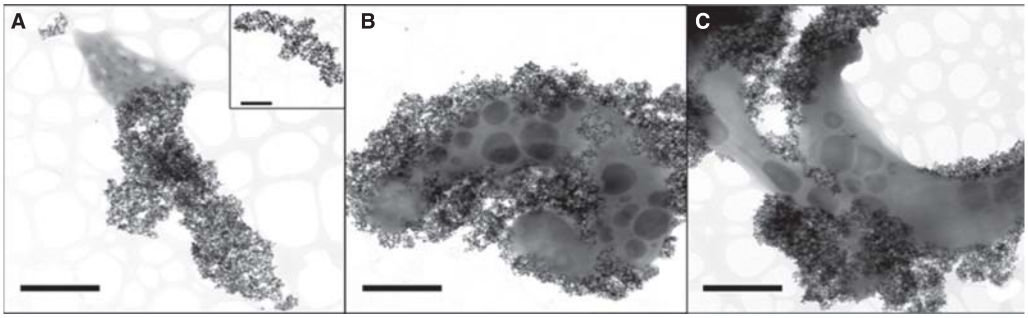

Figure 6. TEM images of algal cells with high degree of attached TiO2 nanoparticle after 24 h (A), 48 h (B) and 72 h (C) exposure to 35 mg/L (scale bars are 2 μm).

seen to form hetero-aggregates with algal cells. The visual inspection of the TiO_2/algae samples confirmed the presence of larger aggregates, which were in some cases in the μm range i.e., similar to the size of algal cells. The use of cell numbers from haemocytometer cell counting may therefore lead to under-estimation of cell density due to difficulties in distinguishing algal cells encapsulated by TiO_2 nanoparticles. The method was found to be more suitable for the less aggregating Au nanoparticles.

Fluorescence of extracted pigments

As it was the case for the Coulter Counting method, the subtraction of background values for nanoparticle interference in the fluorescence method resulted in erroneous determinations of biomass. This was especially critical for initial biomass determination and was due to fluctuations in nanoparticle background fluorescence values making it difficult to obtain consistent and reliable background values. Hence, as described in *Initial biomass quantification*, the average control culture fluorescence (no nanoparticles added) was assumed to be equal to initial fluorescence value for all test concentrations. Due to the increase in algal pigment after 24 h of incubation the subtraction of background values was less sensitive to fluctuations in nanoparticle fluorescence. Thus, the fluorescence values obtained after 24, 48 and 72 h of incubation were corrected for nanoparticle background fluorescence. The dilution series of nanoparticle without algae, run in parallel, was used for background corrections. This procedure was applied both for TiO_2 and Au nanoparticle tests. Background interference of TiO_2 was furthermore found to be influenced by the duration of resting time after addition of acetone, allowing for particle sedimentation prior to fluorescence measurements. Fluorescence was therefore always measured 48 h after sampling. However, this method was not sufficient to reduce interference from the Au nanoparticle dispersions as these were relatively stable in acetone. Tests on the “Starch Control” solutions revealed that the interference could be largely explained by the reagents added to synthesize and stabilize the Au nanoparticles (starch, glucose and MES). Centrifugation was found to not effectively reduce background noise for either particle type (data not shown), and investigations of additional measures are therefore needed to eliminate particle interference.

Growth curves and effect concentrations

As a result of the problems discussed above, the Coulter Counting method was considered to be the least suitable for biomass quantification in the presence of TiO_2 and Au nanoparticles. In the following, only results of haemocytometer counts and fluorescence measurements will therefore be presented and evaluated.

The development of biomass was followed over a period of 72 h using haemocytometer cell counting and fluorescence of pigment extracts. Growth curves are shown in Figure 2. The initial biomass determination has a strong influence on the growth rate estimation and error in determining the initial biomass is therefore one of the most significant sources of error in the algal test. Based on the growth curves, EC values were estimated (Table III).

Results of the algal test with TiO_2 nanoparticles show a dose-dependent decrease in algal growth using both biomass quantification techniques. For TiO_2 nanoparticle incubated algae cultures a concentration-dependent decrease in cell number was observed yielding an EC_{50} value of 160 mg/L (Table III). However, these effects found by haemocytometer

Table III. Comparison of effect values obtained in algal tests (*P. subcapitata*) of Au and TiO_2 nanoparticle suspensions by two different ways of biomass quantification. All concentrations are in mg/L and 95% confidence intervals are given in brackets.

	Fluorescence		Haemocytometer	
	EC_{10}	EC_{50}	EC_{10}	EC_{50}
Au nanoparticle dispersion with starch/glucose/MES (48 h) [mg/L Au]	18 [14;24]	36* [27;47]	–	–
Au nanoparticle dispersion with starch/glucose/MES (72 h) [mg/L Au]	2.8 [1.2;6.7]	38* [21;68]	9.9 [4.3;23]	83* [27;250]
Starch Control (starch/glucose/MES) (72 h) [mg/L starch]	0.4 [0.2;0.9]	15 [6.8;33]	1.4 [0.8;4.5]	12.6 [9.9;16]
TiO_2 nanoparticle dispersions(48 h) [mg/L TiO_2]	10 [1.9;53]	220 [120;390]	–	–
TiO_2 nanoparticle dispersions (72 h) [mg/L TiO_2]	11 [2.2;52]	200 [110;350]	38 [21;69]	160 [120;210]

*Outside the range of tested concentrations (extrapolated value); -, Not counted.

Table IV. Comparison of selected biomass surrogate parameter quantification techniques for use in algal toxicity tests of nanoparticle effects.

	Nano-specific advantages	Nano-specific disadvantages
Haemocytometer cell counting	For non-aggregating nanoparticles - and in lower concentrations of aggregating and adsorbing nanoparticles - it is possible to identify cells and visually distinguish them from nanoparticle background. Besides information on cell density, visual inspection of the sample allows control of algae cell appearance and contributes to an understanding of cell- particle interactions and particle behavior in the suspension (e.g., adherence, formation of agglomerates)	Generally the method is considered time consuming and partly subjective, which can reduce reproducibility (FAO 2011). For nanoparticles forming cell-sized agglomerates and/or adhering to cell surface it is difficult to distinguish cells from particle agglomerates – especially at higher nanoparticle concentrations and dependent on particle type. This may even further hamper reproducibility and accuracy of the method
Coulter Counter cell counting	Output data includes size distributions, e.g., as volume/mL or number/mL. This method therefore gives additional data compared to pigment extraction on the behavior of nanoparticles and algae in the test system. Response is unaffected by interference from nanoparticle colouring or refraction	The sample is diluted in an isotonic solution (containing, e.g., biocides and alkaline salts (BeckmanCoulter 2010)) which may change the behavior of the nanoparticles. A thorough examination of data is necessary to interpret output data since all counts are likely to be mixtures of particles and algae cells due to formation of 2μm-sized agglomerates/aggregates. The method cannot distinguish particles of different shapes and composition which can otherwise lead to highly erroneous results
Pigment extraction	If certain measures are taken, this method can be a suitable option for testing nanoparticles since the biomass surrogate (i.e., pigment) can be physically separated from particles and whereby biomass concentrations can be determined. This method has been suggested as a way of eliminating interferences from particles by ISO (2006)	The presence of nanoparticles may contribute to background fluorescence. Necessary measures to minimize interference from particle-related fluorescence include using control biomass as initial biomass for all test concentrations. Effort should also be made to ensure sufficient settling before measurements: acceleration of settling process, centrifugation and/or filtration may be needed. Though not a nano-specific issue, it should be highlighted that this method does not take into account that pigment content and composition of the individual algae cells may change (both as a result of culture conditions and exposure to nanoparticles)

cell counting may partly or largely reflect an increased difficulty in visually identifying the algal cells. Hence, despite overlapping confidence intervals between EC_{50} values based on haemocytometer cell counting and fluorescence measurements, it is important to point out that cell counting in haemocytometer was found *not* to be a suitable method for TiO_2 nanoparticles. Based on measurements of pigment fluorescence the EC_{50} value for TiO_2 nanoparticles was found to be 200 mg/L and the highest tested concentration (560 mg/L) resulted in a 70% reduction in growth rate. This is in the high end of TiO_2 nanoparticle EC_{50} value ranges for *P. subcapitata* (~6 – 241 mg/L), as reported in a recent review by Menard *et al.* (2011), as well as compared to what we have previously observed for the same type of TiO_2 nanoparticles in our laboratory (Hartmann et al. 2010). Pigment content of three middle concentrations (70–280 mg/L) is not affected by the increase in concentration giving rise to quite large confidence intervals.

Au nanoparticle dispersion exposure caused an inhibitory effect in the highest concentration tested (30 mg/L Au nanoparticles) but did not reach 50% growth inhibition. Up until 48 h, algal cultures in the remaining test concentrations follow the growth of the control sample – both with respect to cell number and pigment content. At 72 h a leveling-off in pigment content of all algal cultures exposed to Au nanoparticle dispersions is seen, except for the lowest concentration (1.9 mg/L) which had a slightly higher growth rate compared to the control. A stimulating effect of emulsifiers may stimulate algal growth due to release of CO_2 or other nutrients during degradation (ISO 2006). The leveling-off in pigment is also reflected in the decrease in EC_{10} for 72 h compared to 48 h. Cell number growth rates did not show the same trend. Together these results indicate that pigment (such as chlorophyll and carotenoid) synthesis is affected by the exposure to Au nanoparticle dispersions despite the continued exponential cell growth. This tendency was confirmed in two additional repetitions of the test. It should be noted that EC_{50} values for Au nanoparticle dispersions are higher than the highest tested concentration, which means that these values should be interpreted with caution. Subsequent tests of the effects of the "Starch Control", however, revealed that the dispersion constituents, and not Au nanoparticles themselves, were largely responsible for both inhibitory effects and the characteristic levelling-off in pigment content after 48 h (see Table III and Supporting information).

Discussion

The use of standard test methods for algal growth inhibition tests (ISO 8692:2002 and OECD 201) has been reported in several studies of nanoparticle toxicity (e.g., Hartmann *et al.* 2010; Hund-Rinke *et al.* 2010; van Hoecke *et al.* 2009). Though it is possible to obtain dose-response relations for the tested nanoparticles the meaningfulness of these results can be questioned due to large uncertainties in test method procedures. This fact is supported by the very large variations in test results from seemingly comparable tests as discussed by Menard *et al.* (2011). This therefore questions the robustness of the applied test methods which is one of the fundamental principles of a standard test guideline, which has the aim of ensuring comparable results (Rand *et al.* 1995).

The results presented in this paper show that choice of algal biomass quantification method in itself can represent a challenge which may be of high importance when testing the effects of nanoparticles. Not only are some quantification methods better suited to cope with the presence of solid particles, but the detection of different types of effects may also depend on the specific choice of biomass quantification method. Reliable measurement of biomass (or surrogate parameters) is the premise for estimation of EC values from standard algal tests. Therefore, it is of outmost importance to identify artefacts that can lead to errors in biomass quantification caused by the presence of particles in the test suspensions and/or particle interactions with algal cells. In general, fluorescence of extracted algal pigments proved to perform better than the other two methods tested as it allows for a physical separation of pigment extract and nanoparticles. However, further refinement of the fluorescence method is needed to eliminate particle background interference. This was especially seen for Au NPs which did not readily settle out after acetone addition in the sample vials and could not easily be removed by centrifugation.

The results shown in this paper underline the advantage of comparing results from several quantification methods. Specifically it resulted in the detection of pigment content leveling off in the algae after 48 h incubation while cell density continued to increase. Absence of algal pigment at higher test concentrations of CeO_2 has also been observed by van Hoecke *et al.* (2009). In another study, a 96 h exposure of *Scenedesmus obliquus* to SiO_2 nanoparticles (10 – 20 nm, 25–200 mg/L) resulted in a concentration-dependent decreased cell content of chlorophyll, whereas the carotenoid content was unaffected. This was hypothesized to indicate shading on a cellular level (Wei *et al.* 2010). Content and composition of carotenoid is known to be affected as a result of photoacclimation (Dubinsky & Stambler 2009). Hence, detailed investigations of such changes may assist to elucidate the effect mechanisms of nanoparticles towards algae. However, in the present study the changes in pigment content caused by Au nanoparticles seemed to be related to the starch/glucose/MES used to stabilize the nanoparticle suspension and hence not caused primarily by the Au nanoparticles themselves. It has been suggested by Handy *et al.* (2012a) that promising approaches to obtain additional information on mechanisms include different fluorescence techniques such as staining with fluoro-chromes combined with detection by flow cytometry as well as investigation of cellular constituents by fluorescence microscopy.

Some of the advantages and disadvantages of using various techniques for quantification of biomass in algal tests of nanoparticle effects are described in Table IV. The three tested methods all have their specific and very different advantages. Counting in a haemocytometer allows for a visual inspection of the cells, Coulter Counting is fast and unaffected by coloring of samples and pigment extraction allows for a physical separation of nanoparticles and biomass surrogate. The interference from particles or other dispersion constituents in fluorescence measurements may be reduced by addition of salts or adjustment of pH (to accelerate sedimentation by causing aggregation/agglomeration) and/or by filtration. In this study centrifugation was found not to effectively reduce interference. Aruoja *et al.* (2009) described that TiO_2, CuO, and ZnO nanoparticles did not cause metal oxide fluorescence when pigments in exposed algae cultures were extracted with ethanol after which fluorescence was measured with a microplate fluorometer (excitation 440 nm, emission 670 nm). Also addition of enzymes or acids, able to degrade starch (Bergmann *et al.* 1988; Pirt & Whelan 1951) may enhance the settling of Au nanoparticles. Future studies may assist to optimise protocols for pigment extraction and quantification and in finding ways of eliminating particle background. The inability to distinguish particles from cells as well as changes in particle aggregation/agglomeration, resulting from dilution in isotonic water in the Coulter Counting methods, makes interpretation of the results difficult. Though this method has been successfully applied in some studies (van Hoecke *et al.* 2009; van Hoecke et al. 2008) the applicability of the method will be limited to nanoparticles where accurate subtraction of background values can be performed or to nanoparticles which are toxic in low concentrations and the particle background is minimal. In our study these methods were found not to be appropriate for either of the tested nanoparticles (Au and TiO_2).

Besides these commonly used methods, which are also described by ISO (2006) several alternatives exist of which a few will be discussed in the following. Dry weight measurements would theoretically be an attractive method for biomass quantification in nano-algal tests. Large scale tests where biomass can be determined by dry weight, would allow for subtraction of nanoparticle background on a mass basis. Biomodification of nanoparticles and attachment to cell surfaces does not influence biomass quantification. However, this method requires very large sample volumes which is problematic both from an economical and practical point of view (Arensberg et al. 1995). For relatively non-toxic nanoparticles, and hence testing of high particle concentrations, a large particle-to-algae dry weight ratio would decrease the accuracy and precision of biomass quantifications. Also the growth conditions can potentially be affected due to insufficient and varying light in large volume containers unless specific measures are taken.

Finally, based on the measured growth inhibition caused by TiO_2 and Au nanoparticles, the following issues were identified:

- For both types of nanoparticles relatively high concentrations (>30 mg/L Au nanoparticles and ~200 mg/L TiO2 nanoparticles) were required to cause 50% reduction in algal growth. For TiO_2 this value is in the high end compared to other values reported (as reviewed by Menard *et al.* 2011) as well as compared to what we have previously observed for the same type of TiO_2 nanoparticles in our laboratory (Hartmann *et al.* 2010). The high variability in observed toxicity of TiO_2 nanoparticles has been discussed by Menard *et al.* (2011) but without reaching any clear conclusions. Until the underlying effects mechanisms are determined it is difficult to compare and evaluate these studies. At present, differences may be attributed to differences in particle characteristics, suspension preparations and incubation conditions resulting in different values of one or more "critical" parameters, determining toxic effects.

- Transformation of Au nanoparticle size distributions and interactions with algal cells occurred during the incubation period resulting in formation of larger agglomerates/aggregates. The particle transformations are thought to be caused by degradation of the starch coating. Also algal exudates are known to increase aggregation (Koukal *et al.* 2007). However, disappearance of Au particles <2 μm at 48 h was also observed for samples without algal cells. Together this indicates either abiotic processes or that a general growth of microorganisms (for example, algae cultures almost always contain bacteria (ISO 2006)), thriving from nutrient richness and the favourable light and temperature conditions, is responsible for the transformation. Although not pronounced, other organisms than algae were also visually detected in the samples (bacteria <5% based on light microscopy). Transformation may hence be through biotic or abiotic degradation of starch combined with – or related to –adsorption of nanoparticles to biological surfaces and increased particle aggregation/agglomeration. It was initially speculated that the observed changes in dispersion behavior (indicating changes in nanoparticle properties) during the incubation was linked to the onset of inhibitory effects and be connected to the observed levelling-off in pigment content in the algae after 48 h (Figure 2).
- Subsequent tests of the effects of the "Starch Control" revealed, however, that other dispersion constituents than Au nanoparticles were responsible for both the inhibitory effects and the characteristic leveling-off in pigment content after 48 h (Supporting information). Lower EC values were determined for the "Starch Control" compared to the Au nanoparticle dispersions (Table III) possibly due to the fact that the constituents were not associated with Au nanoparticles and therefore more available for interactions with the algae. This strongly underlines the fact that testing of nanoparticle dispersions can be viewed as testing of mixtures rather than testing of an individual compound. Furthermore it points to the fact that appropriate controls are necessary in order to avoid misinterpretation of obtained results.

The results, presented in this paper demonstrate that some biological effects might only be detected by use of specific methods. A combination of methods providing complementary information on biomass (e.g., fluorescence of extracted pigments combined with visual inspection and cell counting) may therefore be able to detect effects that would otherwise have been overlooked or misinterpreted may provide insight into the effect mechanisms of nanoparticles. The latter issue does not only apply to algal tests with nanoparticles, but also to algal growth inhibition tests in general.

Apart from the applicability of methods for biomass determination, other major challenges lie in controlling and describing exposure, both quantitatively and qualitatively. This poses a whole range of other questions related to, e.g., preparation of nanoparticle suspensions to ensure reproducibility. From our results it is seen that preparation procedure seem less important in relation to particle sedimentation (and hence aggregation/agglomeration) in algal media compared to the influence of concentration. This thus entails a fundamental problem in testing nanoparticles in aqueous media: namely that exposure varies not only quantitatively but also qualitatively with increasing nanoparticle mass concentration. How to solve this issue needs to be addressed in future research. Another question is how we take into account the kinetic nature of the test system including the fact that test organisms themselves influence the nanoparticle behavior. Algae exudates have been found to increase aggregation of colloidal particles (Koukal *et al.* 2007), but as we have shown increased aggregation/agglomeration may also be due to modifications, such as new coatings or the disappearance of the coating during the course of the test. For poorly soluble substances, such as metals and inorganic metal compounds, it has been suggested by OECD (2000) and OECD (2001) that information on stability, transformation and changes in concentration should be obtained. For nanoparticles a similar information requirement is needed, not only with regards to changes in concentration but also changes in particle/aggregate size, coating etc. If the test aim is to evaluate the toxicity of the pristine nanoparticle, as well as to reduce changes in particle behavior (aggregation/agglomeration, sedimentation, ion release, biomodifications), short term exposure may be an option. To detect changes in algal growth more sensitive detection methods may be needed which could include measurements of ^{14}C assimilation or cell counting in microcounter devices. Supplementary information based on other biomass surrogates and quantification methods may also aid to further elucidate the effect mechanisms of nanoparticles to algae.

Conclusion

Due to the number of variable parameters in ecotoxicological test systems, the testing of nanoparticles can currently be described as an equation with many unknowns. Nanoparticle behavior in aqueous media is controlled by complex interactions between media composition and particle characteristics—interactions which are not at present entirely understood. As a result of the fundamentally different nature of nanoparticles as discrete entities compared to water soluble chemicals the current standard test guidelines are at present inadequate for testing of not readily soluble nanoparticles. One of the major challenges arises from changes in exposure conditions over the test period. Inconsistent exposures are not only due to reduced concentrations (which may occur due to sedimentation or sorption) but also due to changes in particle sizes, removal of coatings etc. Hence it is of utmost importance to monitor both quantitative and qualitative changes in exposure which may help to correctly interpret test results. In algae tests, quantification of biomass is still another challenge. The most common methods used as a surrogate for biomass are based on cell counting (Coulter particle counting and visually in haemocytometer) or fluorescence measurements of pigment extraction (fluorescence). These methods are both hampered by the nanoparticle background, which can be very difficult to subtract due to algae-particle interactions (biotransformation and aggregation/agglomeration formation) and nanoparticle transformation. Based on the findings in this study fluorescence of pigment extracts was found to be the most suitable method as it allows for a physical separation of biomass surrogate (pigment) and particles. However, the method can be adjusted further to reduce particle background noise. Also the combination with visual cell counting is recommended at present as it may help increase our understanding of particle effects and their interactions with the cells. Another path is to further investigate alternative methods to evaluate algae growth such as short term tests using more sensitive endpoints besides growth. Further work is needed to understand the effect mechanisms of Au and TiO_2 nanoparticles towards algae cells and the influence of Au nanoparticle starch coating. The synthesis methods and use of stabilization agents in the production of stable Au nanoparticle dispersions leads to testing of complex products rather than exclusively nanoparticles which influences the interpretation of test results. This emphasizes the fact that appropriate controls are necessary in order to avoid misinterpretation of results.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Figures S.1–S.2.

Notice of Correction

The Early Online version of this article published online ahead of print on 16 August 2012 contained an error on page 5. Figure 3 was incorrect. This has been corrected for the current version.