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Testing particles using the algal growth inhibition test (OECD 201): the suitability of in vivo chlorophyll fluorescence measurements

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Abstract

Background: The freshwater algae and cyanobacteria growth inhibition test (OECD test guideline 201) is frequently used to assess the ecotoxicity of chemicals or particles. A central issue is the measurement of algal growth by quantifying algal biomass over time. Chlorophyll fluorescence measurements are recommended for the testing of particles. The analysis of in vivo fluorescence is the simplest and fastest approach, but is only suitable if there is no interference with the materials. Therefore, in vitro fluorescence analysis is often preferred. We carried out a comprehensive comparison of chlorophyll fluorescence measurements in vitro and in vivo to evaluate the suitability of rapid in vivo testing for the determination of *Raphidocelis subcapitata* biomass in the presence of diverse particles.

Results: For the in vitro measurement, we applied a method that separates particles from chlorophyll using locust bean gum. We tested inorganic and organic particles (including alloys and polymers), ion-releasing and non-releasing materials, and particle sizes in the nanometer to micrometer range with a variety of shapes (spherical, flaky and fibrous). Some of the materials were nontoxic, whereas others showed varying degrees of toxicity (E_rC_{50} = 0.2–100 mg/L in both methods). There were only minor differences between the methods in E_rC_{50} values and the percent inhibition at various test concentrations, but the confidence intervals for the E_rC_{50} values in vivo were narrower and were covered by the range observed in vitro. The in vivo approach showed no limitations, whereas the validity criteria listed in OECD test guideline 201 were not always fulfilled by the in vitro measurements.

Conclusion: The in vivo approach was a suitable and time-saving method for a wide range of particles, although we cannot completely exclude the possibility that some particles may interfere with fluorescence measurement. To avoid false assessments, pre-tests with simple measurements are therefore recommended.

Keywords: Particles, Ecotoxicity, Algal biomass, Measuring method, In vivo fluorescence, In vitro fluorescence

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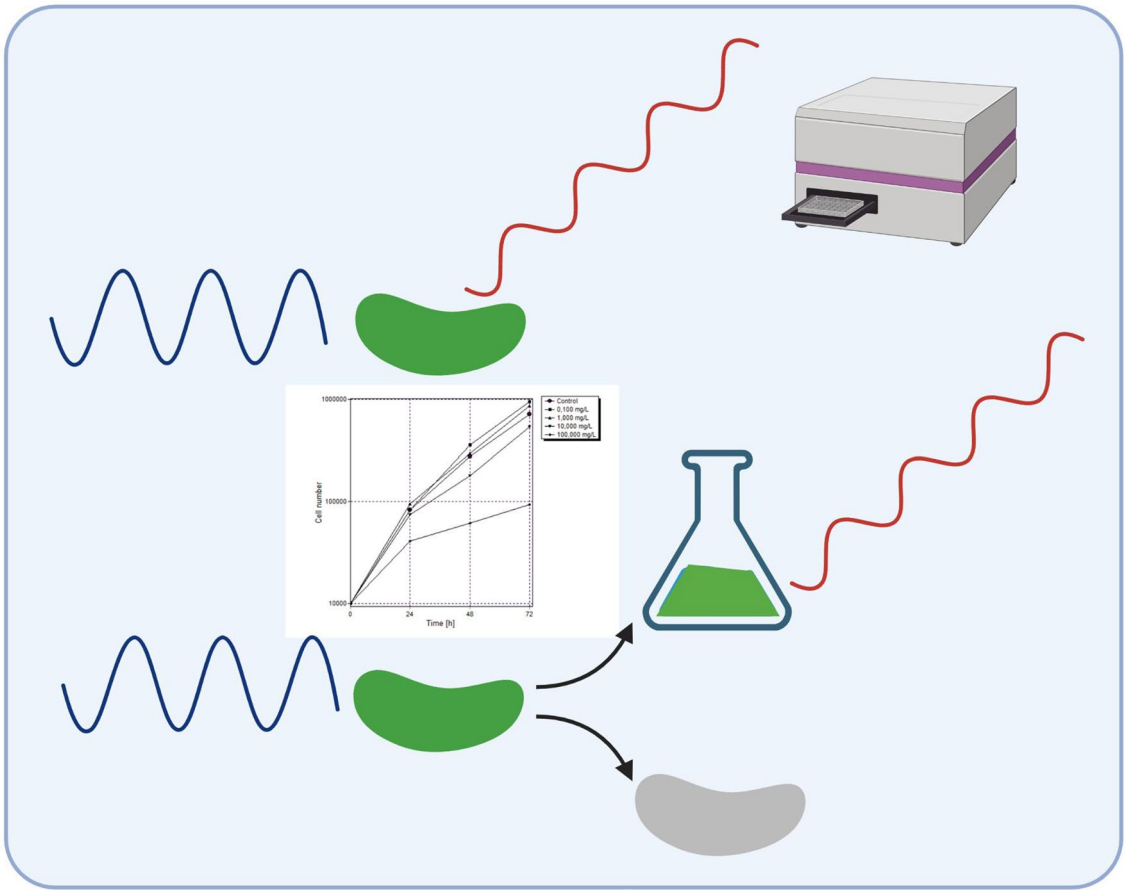
Graphical abstract



Background

The freshwater algae and cyanobacteria growth inhibition test according to OECD test guideline (TG) 201 [1] is often used to assess the ecotoxicity of chemicals and is required by regulations such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) [2]. Specific guidance is provided for the testing of nanomaterials [3, 4] and the Horizon 2020 NanoHarmony project supports the development of such test guidelines and guidance documents (https://nanoharmony.eu/). Larger particles must also be considered, as in the context of microplastics [5]. A central issue is the measurement of algal growth by quantifying algal biomass over time. In OECD TG 201, which was developed for the testing of soluble chemicals, the cell number as an indicator of algal biomass can be determined using an electronic particle counter, microscope and counting chamber, or flow cytometer. Furthermore, biomass surrogates such as pigments or chlorophyll fluorescence are suitable parameters, measured using a fluorimeter, spectrophotometer or colorimeter. However, not all methods are suitable when particles are present. The chemical identity of the particles must also be considered. For some carbon nanomaterials, fluorescence-based chlorophyll quantification methods were shown to be unsuitable for ecotoxicity testing, for example due to shading by multiwall carbon nanotubes and the autofluorescence of graphene oxide [6]. Furthermore, the particle size can affect the suitability of particle counters if the sizes of the algae and particles or their agglomerates are comparable. Manual counting by microscopy is time consuming.

The growth of algae in the presence of particles can be assessed by quantifying algal biomass, usually by measuring chlorophyll fluorescence in vitro or in vivo. However,

the particles may interfere with in vivo measurement and therefore an in vitro method is often preferred [7, 8]. For in vitro analysis, chlorophyll is extracted using agents such as acetone or ethanol [7–10]. In some of these approaches, cells and particles are separated by filtration or flocculation and sedimentation. Fluorescence can be determined rapidly if aliquots of the chlorophyll-containing samples are transferred to microplates and measured using a microplate reader. For the in vivo method, representative samples of the test dispersion containing algae and particles are transferred directly to microplates [11].

Studies in the context of regulation are only accepted if the validity criteria listed in the test guidelines are fulfilled. Three criteria are listed in OECD TG 201 addressing the biomass increase and the growth rate in the control setups.

We generated systematic data to determine the comparative suitability of the in vivo method and an in vitro method for the measurement of algal biomass in the presence of particles. This will support activities for the adaptation of the test guidelines for the growth inhibition test with *Raphidocelis subcapitata*. We conducted in vivo fluorescence analysis, which is advantageous due to the low workload and ease of handling, but is rarely deployed to avoid potential interactions with particles. The results were compared with in vitro fluorescence analysis, which involves the separation of cells and particles and is thus more labor intensive, but excludes any interference phenomena caused by the particles. Testing particles with different chemical identities, shapes and sizes provides information on the suitability of the testing methods for particles with diverse properties.

Materials and methods

Test materials

The diverse particulate test materials are listed in Table 1 and are described in detail in Additional file 1: SI 1. Selection criteria included industrial and economic relevance, as well as availability in different sizes and shapes (fibers, spheres and platelets). We included organic and inorganic materials, pure substances and complex alloys. The polymers differed in terms of backbone structures, aromatic groups and degrees of crosslinking. Given this diversity, we anticipated that some particles would be likely to interfere with the measurement of algal biomass.

Preparation of test dispersions

The preparation method depended on the physicochemical properties of the test material, including hydrophobicity, shape and availability as a dispersion or as a powder. The test concentrations were prepared as a tenfold dilution series. The number of test concentrations was selected according to the expected ecotoxicity, with 100 mg/L as the maximum.

For Ag-ES-4, IN718, Ti64, IN_Y_2O_3 and Ti64_SiC (available as solid powders), we used the dispersion method developed in the project EU-nanOxiMet [12] and adapted in the project nanoGRAVUR [13] for ecotoxicological testing. Briefly, a stock suspension of the particles was prepared by mixing 40 ± 4 mg of the powder with 40 mL ultra-high-quality water to reach a concentration of 1 g/L. The vial with the sample was placed 1 cm above the ultrasonic sensor in the middle of a Cup Horn (Bandelin, Germany) for ultrasonic treatment, and 230 mL of deionized water (4 °C) was added to the Cup Horn.

Table 1 The diverse materials used in the algal growth inhibition tests

Material short name	Supplier	State of material	Average primary particle size
Fibers			
Cu_fiber	Novarials	Powder	Ø 228 nm; length 6.1 µm[a]
TiO_2_fiber	Novarials	Powder	Ø 61 nm; length 1.4 µm[a]
SiC_thin	ACS Materials	Powder	Ø 190 nm; length 11.7 µm[a]
Platelets			
Ag-ES-4	DODUKO	Powder	Ø 15–25 µm[b]
Spheres / compact particles			
PMMA (poly(methylmethacrylate))	Polysciences	Dispersion (2.5 wt%)	Ø 0.3 µm[b]
PE_broad_distribution (polyethylene)	Cospheric	Powder	Ø 0.2–9.9 µm[b]
PA-6 (polyamide)	BASF SE	Powder	Ø 42.2 µm[c]
IN718 (Ni alloy)	ZOZ	Powder	Ø 10.8 ± 9.2 µm[a]
Ti64 (Ti alloy)	ZOZ	Powder	Ø 17.7 ± 14.4 µm [a]
IN-Y_2O_3 (complex alloy composite)	ZOZ	Powder	Ø 26.1 ± 12.5 µm; thickness 3.6 ± 3.1 µm[a]
Ti64-SiC (complex alloy composite)	ZOZ	Powder	Ø 37.9 ± 12.6 µm; thickness 11.0 ± 6.3 µm[a]

[a] Size determined by scanning electron microscopy. [b]Supplier data. [c]Particle size distribution determined using a Mastersizer 3000 (MV Hydro unit); Dx50 presented

The suspension was sonicated for 10 min using a pulse of two (0.2 s/0.8 s). To achieve a sufficient test dispersion volume, two dispersions of 40 mL were prepared and combined. For the highest test concentration (100 mg/L), 50 mL of the test dispersion was added to 450 mL of the OECD test medium and manually shaken for 1 min to ensure the test dispersion was sufficiently homogenous. Concentrations of 10, 1.0 and 0.1 mg/L were prepared by serial dilution.

PMMA was already available as a 2.5 wt% dispersion so we added 2 mL of the dispersion to 498 mL of the OECD test medium to achieve a final stock concentration of 100 mg/L. The stock was gently shaken for ~ 1 min, resulting in a homogeneous and stable dispersion which was immediately used to prepare the remaining test dispersions. The stock dispersion, corresponding to the highest test concentration, was diluted with OECD test medium as described above to prepare test concentrations of 100, 10, 1.0 and 0.1 mg/L.

PE_broad_distribution and PA-6 were available as powders, and a stock dispersion was prepared by mixing 100 mg with 50 mL of the OECD test medium and stirring with a magnetic bar. To achieve concentrations of 100 and 10 mg/L, appropriate aliquots of the homogeneous stock dispersion were diluted with additional test medium.

Cu, TiO_2 and SiC fibers were not processed using the Cup Horn device to avoid a significant reduction in the fiber length. Shaking or stirring were not sufficient to disperse these materials. Therefore, we added 50 mg of the material to 500 mL OECD test medium in a glass bottle (concentration = 100 mg/L) and applied short pulses in a Bandelin Sonorex Type RK 510 ultrasonic bath (HF frequency = 35 kHz, pulse = 10 s, interval = 10 s). Between the short pulses, the dispersion was gently shaken to distribute the particles and sufficient homogeneity was achieved after six pulses. The stock dispersion, corresponding to the highest test concentration, was serially diluted with the OECD test medium to 10, 1.0 and 0.1 mg/L.

Ecotoxicological assay

The inhibition of algal growth (*R. subcapitata*) was measured as described in OECD TG 201 [1], using the prescribed OECD growth medium. During the test, all vessels (250-mL conical glass flasks containing 100 mL test dispersion) were maintained at 21–24 °C (controlled at ± 2 °C). The test was performed with a light intensity (OSRAM Standard cool white bulbs) of ~ 100 µE m^{-2} s^{-1} (4440–8880 lx). The light intensity was measured daily using an LI-250A cosine (2 π) receptor (LI-COR BioSciences, USA) at the level of the test medium surface and at different positions in the incubation chamber. During the test, all vessels were placed on an Incubation Shaker Multitron (INFORS-HT, Switzerland) shaking continuously at 150 rpm. The test culture temperature was measured in one additional test vessel in total. At the start of the test, all test vessels were randomly placed in the incubator and randomly repositioned daily thereafter. For the growth test, we prepared four replicates of each test concentration and six replicates of the control. The test vessels were filled with 100 mL of the test dispersion containing the particulate test material. The blank control consisted of fresh growth medium only. The cell density of the inoculum culture (pre-culture) was determined using a CASY Model TT cell counter (Roche Innovatis, Germany) and an aliquot of the inoculum culture (423–1105 µL) was added to the test vessels to obtain a density of 10,000 cells/mL, as recommended in OECD TG 201 [1]. The cell density in the test vessels was determined by fluorescence measurements in vivo and in vitro after 24, 48 and 72 h.

For the in vivo fluorescence measurements, four 200-µL replicates of each test vessel were transferred to 96-well plates and the cell density was determined by measuring the fluorescence on a Synergy MX microplate photometer (BioTek, Germany). The excitation wavelength was 440 nm and the emission wavelength was 690 nm. For the in vitro assay, the cell density was determined by direct fluorescence analysis of extracted chlorophyll. The method was developed by one of the partners in the joint project MARINA (funded by the EU Seventh Framework Programme), resulting in a joint publication proposing modifications for eight guidelines including OECD TG 201 [8]. For the in vitro fluorescence measurement, 150 mg of locust bean gum (Sigma-Aldrich, USA) was dissolved in 100 mL deionized water at 100 °C by stirring. We also prepared a dispersion of acetone containing 0.1% $MgCO_3$. We transferred 1-mL samples from each test vessel into individual 15-mL screw-cap tubes and added 0.1 mL locust bean gum solution and 4.4 mL of the acetone/$MgCO_3$ dispersion to precipitate the particles. The mixtures were shaken and left for 1 day in the dark at room temperature before measuring the fluorescence signal as above.

Calibration curves were prepared for both methods correlating fluorescence signals to cell counts (Additional file 1: SI 2). Test validity was confirmed by the following control criteria: biomass increase in the control cultures ≥ factor 16, mean coefficient of variation for section-by-section specific growth rates ≤ 35%, and coefficient of variation of the average specific growth rates ≤ 7%. The results for the positive control (3,5-dichlorophenol) are provided in Additional file 1: SI 3.

Statistics

ToxRat (ToxRat Solutions, Germany) was used to evaluate effect concentrations and to confirm the fulfilment of validity criteria. We calculated the percentage inhibition of the growth rate [r] compared to controls for the entire exposure period. The biological data were analyzed to determine E_rC_{50} values together with 95% confidence intervals, where possible.

Results

We applied the in vitro and in vivo methods concurrently to the same test dispersions to allow the direct comparison of results. The particles affected algal growth to varying degrees, ranging from no inhibition to complete inhibition. Table 2 shows the E_rC_{50} values for the growth rate as well as the degree of inhibition relative to the control at each test concentration. We observed only

Table 2 In vitro and in vivo chlorophyll measurements for nanoparticles and microparticles, focusing on E_rC_{50} values and the degree of inhibition relative to the control at each test concentration

Material	Method	E_rC_{50} [mg/L][a]	Effect [%][b]			
			0.1	1	10	100
Fibers						
Cu	In vitro	0.16 [0.07–0.34]	39	88	100	100
	In vivo	0.28 [0.19–0.42]	28	76	100	100
TiO_2_fibers	In vitro	1.3 [n.d.][b]	10	29	100	100
	In vivo	1.9 [0.7–5.1]	12	35	82	85
SiC_thin	In vitro	99.9 [29.1–315.6]	− 7	− 4	9	48
	In vivo	89.4 [45.3–169.5]	− 2	4	12	53
Platelets						
Ag—ES-4	In vitro	10–100	12.4	2.5	− 0.6	100
	In vivo	10–100	0.1	3.9	− 1.0	100
Spheres / compact particles						
PMMA	In vitro	No effect	n.i.[c]	− 4.9	− 1.7	− 4.8
	In vivo	No effect	n.i.[c]	1.7	6.2	2.7
PE_broad_ distribution	In vitro	No effect	n.i.[c]	n.i.[c]	24.1	47.2
	In vivo	No effect	n.i.[c]	n.i.[c]	15.5	41.4
PA-6	In vitro	No effect	n.i.[c]	n.i.[c]	− 4.8	− 6.0
	In vivo	No effect	n.i.[c]	n.i.[c]	− 3.7	− 4.4
IN718	In vitro	No effect	27.9	23.5	27.0	21.2
	In vivo	No effect	20.3	18.4	22.0	16.2
Ti64	In vitro	No effect	− 0.1	16.5	− 0.9	− 6.5
	In vivo	No effect	1.0	4.6	1.4	− 1.5
IN-Y_2O_3	In vitro	No effect	n.i.[c]	− 13.3	− 7.3	− 22.0
	In vivo	No effect	n.i.[c]	− 12.9	− 5.9	− 16.6
Ti64-SiC	In vitro	No effect	1.0	− 3.9	− 13.9	− 15.3
	In vivo	No effect	− 1.7	− 2.3	− 6.1	− 2.5

[a] Due to the dilution factor of 10, the E_rC_{50} value is just a proxy for a rough assessment of ecotoxicity; values in square brackets represent confidence intervals. [b]Mean inhibitory effect at various test concentrations; n.d. = not determinable for mathematical reasons. [c]n.i. = not investigated

(See figure on next page.)

Fig. 1 In vitro and in vivo chlorophyll measurements for nanoparticles and microparticles, focusing on compliance with the OECD TG 201 validity criteria. **A** Validity criterion "biomass increase within the 72-h test period" threshold value ≥ 16. **B** Validity criterion "mean coefficient of variation (CoV) for section-by-section specific growth rates" threshold value ≤ 35%. **C** Validity criterion "coefficient of variation (CoV) of average specific growth rates" threshold value ≤ 7%. Solid line: validity criterion (threshold value). 1 Cu_fibre; 2 TiO_2_fibers; 3 SiC_thin; 4 Ag-ES-4; 5 PMMA; 6 PE_broad distribution; 7 PA-6; 8 IN718; 9 Ti64; 10 IN-Y_2O_3; 11 Ti64-SiC

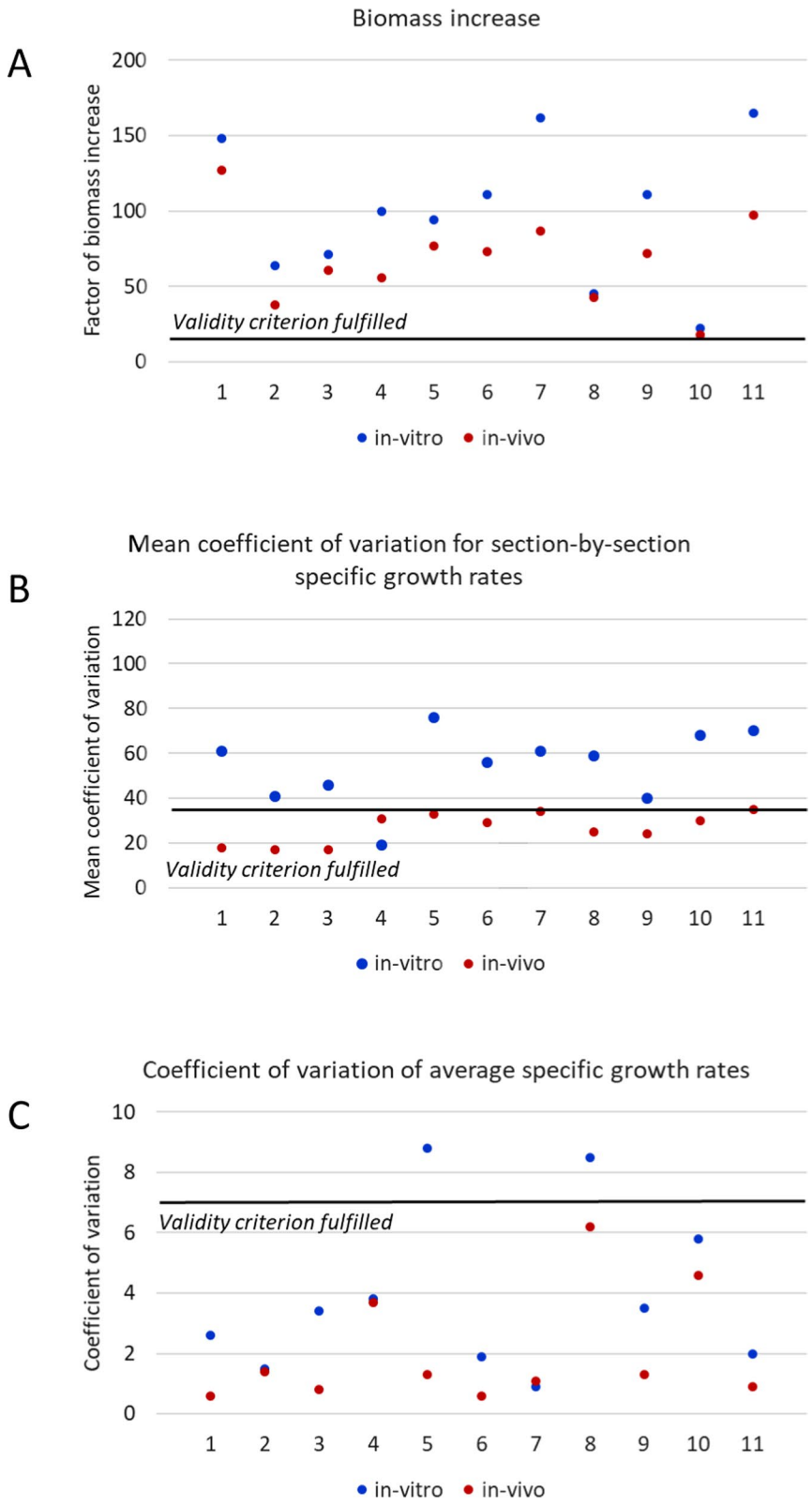


Fig. 1 (See legend on previous page.)

small differences between the methods in terms of the E_rC_{50} values and percent inhibition at various test concentrations, but the confidence intervals for the E_rC_{50} values were smaller in vivo and were covered by the range observed in vitro. Figure 1 indicates how well the in vitro and in vivo methods comply with the OECD TG 201 validity criteria. Only the in vivo method fulfilled all validity criteria. In the in vitro method, the mean coefficient of variation for section-by-section specific growth rates should be $\leq 35\%$, but this was achieved only for the test with Ag-ES-4. Furthermore, the coefficient of variation for the average specific growth rates in the controls exceeded the threshold value of 7% in the tests with two spherical materials (PMMA and IN718).

The biomass in the control cultures should increase exponentially by a factor of at least 16 during the 72-h test period. In all experiments, the increase was higher when the biomass was determined in vitro following chlorophyll extraction. The biomass increase factor was 22–165 for the in vitro measurements and 18–127 for

Table 3 Coefficient of variation (CV) of cell numbers at the end of the test (72 h) for in vitro and in vivo measurements (absolute values as well as the ratio of the in vivo and in vitro values)

Material	Chlorophyll measurement	Control	0.1 mg/L	1 mg/L	10 mg/L	1000 mg/L
Fibers						
Cu	CV in vitro	12.7	65.6	39.2	–[a]	–[a]
	CV in vivo	2.7	46.1	4.6	–[a]	–[a]
	Ratio in vitro/in vivo	4.7	1.4	8.5	–[a]	–[a]
TiO_2_fibers	CV in vitro	6.4	18.3	34.8	1.2	3.3
	CV in vivo	1.2	2.3	1.5	0.1	0.3
	Ratio in vitro/in vivo	81.2	43.7	67.7	760.1	305.2
SiC_thin	CV in vitro	13.8	14.0	33.5	55.8	32.3
	CV in vivo	3.4	18.9	13.9	34.6	5.7
	Ratio in vitro/in vivo	4.1	0.7	2.4	1.6	5.7
Platelets						
Ag-ES-4	CV in vitro	18.1	17.8	20.0	16.1	0.0
	CV in vivo	14.9	17.3	15.5	4.0	0.0
	Ratio in vitro/in vivo	1.2	1.0	1.3	4.0	0.0
Spheres / compact particles						
PMMA	CV in vitro	36.6	n.d.[b]	15.4	7.0	31.2
	CV in vivo	5.7	n.d.[b]	6.3	5.1	8.4
	Ratio in vitro/in vivo	6.4	–[a]	2.4	1.4	3.7
PE_broad distribution	CV in vitro	8.5	n.d.[b]	n.d.[b]	17.7	11.5
	CV in vivo	2.7	n.d.[b]	n.d.[b]	5.3	3.9
	Ratio in vitro/in vivo	3.1	–[a]	–[a]	3.3	2.9
PA-6	CV in vitro	4.4	n.d.[b]	n.d.[b]	12.1	10.2
	CV in vivo	4.7	n.d.[b]	n.d.[b]	2.5	9.1
	Ratio in vitro/in vivo	0.9	–[a]	–[a]	4.8	1.1
IN718	CV in vitro	30.3	51.1	3.2	42.2	33.8
	CV in vivo	23.1	45.3	9.0	40.3	38.4
	Ratio in vitro/in vivo	1.3	1.1	0.4	1.0	0.9
Ti64	CV in vitro	15.8	17.5	21.9	25.5	11.0
	CV in vivo	5.3	17.8	13.2	18.2	6.9
	Ratio in vitro/in vivo	3.0	1.0	1.7	1.4	1.6
IN-Y_2O_3	CV in vitro	18.4	n.d.[b]	6.9	10.2	7.0
	CV in vivo	13.4	n.d.[b]	6.8	9.4	2.4
	Ratio in vitro/in vivo	1.4	n.d.[b]	1.0	1.1	2.9
Ti64-SiC	CV in vitro	10.3	36.8	22.8	9.7	4.5
	CV in vivo	4.1	4.2	4.2	4.3	3.0
	Ratio in vitro/in vivo	2.5	8.8	5.4	2.3	1.5

[a] 100% toxicity; no calculation possible. [b] n.d. = not determined

the in vivo measurements. Furthermore, as an additional parameter, we calculated the coefficient of variance of the cell number at the end of the test (Table 3). In 95% of the experiments, the coefficient for the replicates at every test concentration and the control was smaller for the in vivo measurement. The exceptions were TiO_2 fibers (10 and 100 mg/L), the SiC thin fiber (0.1 mg/L), IN718 (100 mg/L) and the PA-6 control.

Discussion

Information on growth inhibition

The chlorophyll content is a good indicator of algal biomass, and both in vivo and in vitro fluorescence measurements are recommended in OECD and ISO guidelines for the algal growth test, including OECD TG 201 [1] and EN ISO 8692 [14]. The latter recommends in vivo measurements in the presence of 3-(3,4 dichloro-phenyl)-1,1-dimethylurea to obtain the maximum fluorescence yield [15]. Other guidelines such as DIN 38412-33 [16] do not mention the addition of this herbicide.

We found that both in vivo and in vitro fluorescence measurements were suitable for the quantification of chlorophyll and thus the determination of algal biomass, resulting in a comparable assessment of diverse particulate test materials regardless of their chemical identity, shape and size. We tested inorganic and organic materials, materials in the nanometer and micrometer ranges, as well as spherical, flaky and fibrous materials. Some of these materials were nontoxic but others showed various degrees of toxicity. Given the dilution factor of 10 between the tested concentrations, the E_rC_{50} values for growth rate can be considered as a proxy. According to OECD TG 201, the dilution factor should not exceed 3.2. Therefore, we also presented the percentage inhibition at the individual test concentrations. We observed reliable concentration–effect relationships, in contrast to Hartmann et al. [17]. That study of three TiO_2 nanoparticles revealed scattered effect values for one of the particles, and only a trend regarding the potential toxicity of this material. The cause of the divergent behavior of this material remained unclear, but extensive agglomeration of the material in the test medium may have been responsible.

Validity of the tests

Although in vivo and in vitro chlorophyll fluorescence measurements are both recommended by OECD and ISO guidelines for the algal growth inhibition test, we found that the validity criteria were not always fulfilled by the in vitro measurements. The validity criteria refer to control treatments without particles, thus excluding any influence of the particles on the fluorescence signal.

The validity criterion providing information on the daily growth rate (mean coefficient of variation for section-by-section specific growth rates) failed when determined by in vitro fluorescence analysis, with slowed growth after 24 or 48 h. For the Ti64-SiC material, the cell number was determined using a cell counter in additional control treatments. The preparation of the additional treatment included the same inoculum culture, the same starting cell concentration (10,000 cells/mL, measured with a cell counter comparable to the other tests) as well as the same stock solutions for the test medium and the same incubation devices. All validity criteria were fulfilled when using the cell counter. Assuming that control treatments were comparable for the in vivo and in vitro fluorescence measurements and cell counts, the difference could indicate that the relationship between fluorescence and cell number changes during growth when using the chlorophyll extraction method. This would indicate lower extraction efficiencies for higher cell numbers. However, a relationship between fluorescence intensity and validity was not observed in our in vitro measurements. The test performance was highly standardized, but cell growth rates nevertheless differed. Most in vitro measurements resulted in non-valid tests regardless of the cell growth rate. Even tests with a low cell growth rate failed to meet the validity criteria, as demonstrated for IN-Y_2O_3. Importantly, the calibration curve showed a correlation coefficient (R^2) of 0.99 (Additional file 1: SI 2), confirming that the extraction efficiency did not change up to the maximum cell number of 4.6×10^6 cells/mL. This exceeded the maximum cell number in any of our tests, where the maximum (in the test with Ti64-SiC) was 1.6×10^6 (mean of six replicates).

The proposed weakness of the extraction method was supported by the success of the in vivo fluorescence measurements, all of which met the validity criteria. Furthermore, we found that the efficiency of extraction was affected by the algal species in previous experiments. No concentration–effect curves were obtained for *Desmodesmus subspicatus* and several CeO_2 nanoparticles, and the extracts did not meet the validity criteria (data not shown). However, in vivo measurement yielded valid tests with clear concentration–effect relationships. This indicates a need to adapt the extraction method so that it meets the OECD TG 201 validity criteria. In our method, the algae and particles were separated using locust bean gum to promote particle sedimentation, thus avoiding interference with the measurement. Locust bean gum was also added to the controls to ensure equal treatment, which could interfere with chlorophyll extraction. An alternative extraction method without locust bean gum has been developed for *Selenastrum capricornutum* (former name for *R. subcapitata*) [9].

The relationship between in vivo fluorescence and cell numbers has been studied in detail, revealing a linear regression between in vivo fluorescence and the abundance of green algae [18]. This fits with our observation that in vivo fluorescence analysis produces a higher number of valid tests. Previously, the mass increase of *R. subcapitata* determined by the measurement of fluorescence in vivo was shown to be statistically equivalent to values obtained by cell counting and extracted fluorescence, but the extraction method was not described [19]. Because natural water samples were tested, the use of locust bean gum to support the precipitation of particles appears unlikely and may explain the relationship between the in vitro and in vivo chlorophyll measurements. Furthermore, the authors found little variance in the in vivo fluorescence measurements between replicate flasks, agreeing with the smaller coefficients of variation we observed for our in vivo measurements compared to in vitro tests.

Identification of potential interferences

The measurement of in vivo fluorescence is only applicable if there is no interference with the materials and some authors thus prefer in vitro measurements when assessing the ecotoxicity of particles [7]. The inhibition values determined using both methods in our study showed that there was no interference from the materials despite their diverse physicochemical properties, but we cannot fully exclude the possibility of interference with the in vivo fluorescence measurement. There is little information about the potential sources of interference, but short-term pre-tests can confirm the suitability of in vivo fluorescence measurements. They can increase the reliability of the results and avoid unnecessary and time-consuming in vitro testing. A suitable test design is required, and assessment criteria for the results must be defined. One starting point for the development of a pre-test approach is to measure the fluorescence of suspensions containing (i) algae, (ii) test material, and (iii) mixed algae and test material at concentrations representative of the full test. The fluorescence of the suspensions should be measured immediately after preparation to avoid any toxic effects of the materials on the algae. An example is presented in Additional file 1: SI 4. Three potential outcomes are possible (Table 4):

- The fluorescence signal of the suspensions containing (i) algae and (iii) mixed algae and test material are comparable while the suspension containing (ii) the test material matches the background value. Threshold values indicating the comparability of the results are required.
- The fluorescence signal of the suspension containing (i) algae exceeds that of the (iii) mixed algae and test materials. There is no fluorescence in the suspension containing (ii) the test materials. The materials are non-fluorescent, but reduce the fluorescence signal of the algae. Threshold values indicating tolerable differences are required.
- Fluorescence is detected in all three suspensions.

An in vitro measurement would be preferred in cases where there is a decrease or increase in the fluorescence signal (cases 2 and 3 in Table 4). A decrease in the fluorescence intensity may occur (i) if incident light is blocked before it reaches the algal cells, reducing their photosynthetic activity and thus the fluorescence signal, or (ii) if fluorescence emitted from the algae is blocked before it reaches the sensor, which may be due to the absorption by suspended particulate materials or particles that have agglomerated with algal cells and thus obscured the cell surface [6]. Conversely, an increase in the fluorescence signal is most likely caused by autofluorescence, as previously demonstrated for graphene oxide [6]. However, for in vitro analysis to be suitable, it would be necessary to eliminate any shortcomings in the extraction method. An alternative approach is to measure the fluorescence spectrum of the particles in the test medium. The in vivo measurement could then be used for the test in the absence of a signal at the excitation wavelength used for the fluorescence measurement.

In cases where the in vitro test is used, chlorophyll *a* must not be absorbed by the particles. When the particles with adsorbed chlorophyll *a* are separated during extraction, the lower chlorophyll content in the

Table 4 Potential outcomes of pre-tests to determine the suitability of in vivo fluorescence analysis and their interpretation

Case	Suspension containing:			Interpretation	Suitability of in vivo fluorescence
	Algae (i)	Materials (ii)	Algae + materials (iii)		
1	Fluorescence signal	No/low fluorescence signal	Fluorescence signal comparable to (1)	No interference	Yes
2	Fluorescence signal	No / low fluorescence signal	Reduced fluorescence signal compared to (1)	Reduction of fluorescence by materials	No
3	Fluorescence signal	Fluorescence signal	Fluorescence signal	Autofluorescence of materials	No

remaining solution would indicate a false-positive ecotoxic effect on the algae. A quick second screening assay could be used to confirm the suitability of the in vitro method. Following the approach of Farkas and Booth [6], a stock dispersion of the materials could be mixed with the extracted chlorophyll by shaking briefly, then removed by filtration or flocculation and settling. The remaining fluorescence would be measured and compared with the results of a control sample treated in the same way but containing only chlorophyll. A difference of less than 10% would indicate tolerable adsorption and, accordingly, the suitability of the in vitro method.

Conclusions

We investigated the in vivo measurement of fluorescence in the growth test with green algae according to OECD TG 201 to evaluate whether it can determine algal biomass in the presence of particles and deliver results comparable to in vitro measurements following the separation of particles from extracted chlorophyll using locust bean gum. We considered a wide range of potential influencing factors on the two methods by including particles of various shapes (spherical, elongated and flaky), sizes (0.030–250 µm), different chemical identities (metals, metal oxides, organic polymers and inorganic carbon compounds), simple and complex compositions, stable and toxic ion-releasing substances, and wide range of ecotoxicity profiles (toxic with an E_rC_{50} value of 0.16 mg/L up to no growth inhibition). We found that both methods showed comparable results for the inhibition of algal growth, but only the in vivo method consistently fulfilled the validity criteria. We therefore conclude that in vivo fluorescence analysis is a robust and time-saving alternative to the measurement of fluorescence in vitro for the quantification of algal biomass. We observed no interference caused by particle autofluorescence, but interference with the particles cannot be excluded completely and we therefore recommend the development of pre-tests to identify such issues.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-022-00623-1.

Additional file 1: SI 1. Test materials. **SI 2.** Calibration curves: fluorescence intensity – cell number. **SI 3.** Results for the positive control 3,5-dichlorophenol. **SI 4.** Examples using short-term pre-tests to confirm the suitability of in vivo fluorescence measurements.

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Conceptualization, KHR; investigation, RS; writing—original draft preparation, KHR and KS; project administration, KHR; funding acquisition, KHR. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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