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RESEARCH ARTICLE

The Effect of TiO_2 Nanoparticles on the Aquatic Ecosystem: A Comparative Ecotoxicity Study with Test Organisms of Different Trophic Levels

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Abstract

A comprehensive ecotoxicological assessment was carried out with Degussa VP nano TiO_2 suspension applying a bioluminescent bacterium (Aliivibrio fischeri), algae (Pseudokirchneriella subcapitata, Scenedesmus subspicatus and Chlorella vulgaris), a protozoon (Tetrahymena pyriformis), the water flea (Daphnia magna) and an aquatic macrophyte, Lemna minor. TiO_2 nanoparticles were toxic in the set of the conducted tests, but the toxicity level varied with the organisms and endpoints. According to our results the concentrations, the duration and the mechanisms of exposure are contributing factors to the toxicity of nanoparticles. The Tetrahymena phagocytic activity, the Daphnia heartbeat rate and the Lemna total chlorophyll content as ecotoxicity endpoints showed outstanding sensitivity. These organisms showed significant behavioural and physiological changes when exposed to low TiO_2 nanoparticle concentrations (0.1 and 0.05 µg/L) considered to be lower than the predicted environmental concentration in surface waters. These results reveal the importance of behavioural and physiological assays in assessing the impact of nanoparticles and indicate that nanosized TiO_2 may pose risks to the aquatic ecosystem.

Keywords

titanium dioxide nanoparticles, ecotoxicity, aquatic ecosystem, sublethal endpoints

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1 Introduction

The risk assessment and the investigation of the adverse effects of emerging pollutants are common topics in the current literature. These potentially harmful chemical substances can be grouped as pharmaceuticals, detergents, cosmetics, industrial additives and agents, pesticides or nanomaterials [1, 2, 3, 4]. The growth of the environmental concern of nanomaterials is inevitable due to their increased manufacture and widespread use, which results in a need to focus on and explore their impact on the environment [5, 6, 7]. In the near past scientists started to assess the risk of engineered nanoparticles (NPs) in different ecosystems. These investigations are still at an early stage.

Significant contradictions can be found between the results of different authors concerning the toxicity of nanoparticles. The effect of NPs depends on many physico-chemical factors and processes, which all affect the fate and behaviour of NPs [8]. The effect of the particle shape, size, surface area, surface charge on the aggregation properties, nanosize range and ecotoxicity must be considered [9, 10]. An important factor is the adsorption capacity of the NPs onto different surfaces, including the cell boundary surfaces of microorganisms. Finally, we must consider the effects of other abiotic factors such as pH, ionic strength, water hardness, and the naturally occurring organic materials in the test medium [11, 12]. The standard test medium, which is essential for the test organism, may affect the dispersability, aggregation and sedimentation of the tested NPs [13].

The toxic properties of NPs have been revealed by determining the EC_{50}, LC_{50}, NOEC and LOEC values for different aquatic testorganisms [9, 14, 15, 16, 17, 18, 19, 20]. Kahru and Dubourguier [10] analyzed 77 effect values for $nTiO_2$, C60, nZnO, nAg, SWCNT (single-walled carbon nanotube) sand, nCuO and MWCNTs (multi-walled carbon nanotubes). The applied test organisms were mainly crustaceans, bacteria, algae and fish species. They found that nAg and nZnO were the most toxic, $nTiO_2$ was classified as "harmful" with an $L(E)C_{50}$ value 10–100 mg L^{-1}. For all studied organism groups, the dispersability of the different NPs was the key factor in their aquatic toxicity.

In order to gain a satisfactory picture of the environmental effects of NPs, more trials would be necessary with marine test organisms and several freshwater and terrestrial vertebrate and invertebrate species applying physiological responses. Data are also missing for terrestrial plants and other photosynthetic organisms as well [11]. Therefore, there is a need for studies using environmentally relevant test organisms with feasible and sensitive measurement endpoints to reveal the unknown potential adverse effects. The ecotoxicological effects of NPs available in the literature shows high variability even within the same organism group [10].

The unicellular protozoa as test organisms are well applicable to determine the effects and toxicity of NPs on cell function [7]. Protozoa play an important role in maintaining the balance of the ecosystem of microbial life, and they are important food source for larger creatures and the basis of many food chains. Therefore, the toxic effects of NPs on these unicellular organisms have great importance. Rajapakse et al. [21] exposed *Tetrahymena thermophila* cells to $nTiO_2$ for 24 h, which resulted in changes of membrane fatty acid profile, but no lipid peroxidation was detected. They interpreted these changes as acclimation to unfavourable conditions, not as toxic effects.

The algal growth inhibition tests are widely distributed in the risk assessment of waters. *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum* or *Rhapidocelis subcapitata*) and *Desmodesmus subspicatus* are commonly used freshwater algae testorganisms, which are proven to be very sensitive to heavy metal pollution [9, 22]. Ecotoxicity of nanosized TiO_2 on alga have been summarized in several papers in recent years [10, 23]. Extremely variable values were reported for the tested $nTiO_2$, and there was no clear relationship found between the concentrations, primary particle size and toxic effect.

During experiments with *Daphnia magna* $nTiO_2$ was observed on the integument, antennas and in the digestive system of *Daphnia*. If greater quantities of nanoparticle aggregates accumulate on the surface of *Daphnia* it is likely to cause mobility problems [14]. *Daphnia magna* behaviour (motility, feeding frequency) and heart beat rate were not affected during 60 minutes exposure to $nTiO_2$ [24]. The standard deviations are very large between results published by different authors even in case of the same test organism and similar experimental conditions. This is probably because the applied TiO_2 NPs show a very wide range concerning the physico-chemical properties such as particle size, crystal form, coatings, surface area and purity. It seems likely that it is not the primary size of nanoparticles, but the secondary (formed by aggregation) size that affects the toxicity [25].

Kim et al. [26] investigated the toxic properties in *Daphnia* focusing on the activities of four antioxidant enzymes: catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase. They found that mortality was significantly increased at 5 and 10 mg L^{-1} $nTiO_2$ concentration during the chronic bioassays; and the reduction of reproduction ability was not observed. The antioxidant enzyme activities in *D. magna* were increased, but superoxide dismutase did not show a concentration-dependent increase. The size of the applied $nTiO_2$ was <40 nm.

Zhu et al. [27] tested Degussa P25 $nTiO_2$ with an average surface area of 50 m^2/g and particle size of 21 nm (20% rutile; 80% anatase). Acute (72 h) and chronic (21 d) toxicity tests were carried out to investigate the accumulation of $nTiO_2$ in *D. magna*. They found minimal toxicity within 48 h exposure time, but high toxicity was registered within 72 h. In case of 21 d chronic test, *D. magna* displayed severe growth retardation, mortality, and reproductive defects, and a significant amount of $nTiO_2$ was found accumulated in *D. magna*.

Due to the heterogeneity of physico-chemical properties of TiO_2 NPs, it is important in all cases to document the characteristics of the used materials, because the chemical and physico-chemical properties of a material essentially dictate the molecular-level interactions [25, 28].

Since a daunting variety of TiO_2 NPs is produced and applied for ecotoxicity studies, it is complicated to discuss our findings in the reflection of the results of other authors. To support easier perspicuity and understanding, a summarizing table (Table 1) was prepared containing extensive data on the ecotoxic effect of different TiO_2 NPs from current literature applying the same test species as applied in our study.

This paper is a contribution to the general effort towards a better understanding of the ecotoxicity of engineered NPs in the aquatic ecosystem with a special emphasis on sublethal effects.

2 Materials and methods

The ecotoxicity of HCl activated Degussa VP P90 nano TiO_2 was investigated with test organisms from different trophic levels. A marine bioluminescent bacterium (*Aliivibrio fischeri*), algae (*Pseudokirchneriella subcapitata, Scenedesmus subspicatus* and *Chlorella vulgaris*), and a ciliate protozoon *Tetrahymena pyriformis* were applied as unicellular model organisms. The freshwater aquatic macrophyte *Lemna minor* (common duckweed) and a freshwater crustacean *Daphnia magna* (water flea) were used for the determination of adverse effects of $nTiO_2$ on higher trophic levels.

2.1 Applied $nTiO_2$ suspension

Degussa VP P90 $nTiO_2$ powder purchased from Evonik Resource Efficiency GmbH was activated by 0.1 M HCl. During the activation process 3 g of Degussa VP P90 $nTiO_2$ powder and 10 mL of 0.1 M HCl solution were homogenized thoroughly, then sonicated in a 65°C ultrasound water bath in a 100 cm^3 ground-neck round-bottom boiling flask for 15 min. Then the HCl solution was evaporated with a rotary vacuum evaporator merging the flask into a 50°C water bath. To this dry activated $nTiO_2$ powder, 23 mL of MilliQ® ultrapure water was added in small proportions while homogenizing and the suspension was sonicated in a 65°C ultrasound water bath for 20 min.

Table 1 Toxicity data on different types of $nTiO_2$ particles from current literature [mg L^{-1}]

Characteristics of the tested nano-TiO_2	Test organism	Toxicology endpoint	NOEC	LOEC	EC_{10}	EC_{50}	Reference
Primary particle diameter: 25–70 nm	*Aliivibrio fischeri*	Bioluminescence (30 min)	> 20000	–	–	>20000	[17]
Particle size: diameter: ~6 nm	*Aliivibrio fischeri*	Bioluminescence (10 min)	no observed effect between: 0.4395–112.5 mg L^{-1}				[29]
Primary particle diameter: 5–10 nm Surface area: 324 m^2/g Amorphous and crystalline phases: > 99.0% Anatase Zeta-potential: –21.6 mV	*Chlorella vulgaris*	Growth inhibition (6 days)	–	–	*EC_{30}: 30	–	[30]
TiO_2-content: 99.7% Primary particle diameter: < 25 nm Surface area: 200-220 m^2/g	*Chlorella sp.*	Growth inhibition (72 h)	0.89	–	–	16.12	[31]
Coated DLS: ~380 nm Surface area: 5.8 m^2/g	*Pseudokirchneriella subcapitata*	Growth inhibition (72 h)	–	–	–	61	[32]
DLS: 140 nm Surface area: 38.5 m^2/g Amorphous and crystalline phases: 21% Anatase, 79% Rutile TiO_2-content: 90%			–	–	–	87	
Primary particle diameter: < 100 nm TiO_2-content: 99.9%	*Pseudokirchneriella subcapitata*	Growth inhibition (72 h)	–	–	*EC_{25}> 100	–	[15]
Primary particle diameter: 25–70 nm	*Pseudokirchneriella subcapitata*	Growth inhibition (72 h)	–	–	–	5.38	[9]
Primary particle diameter: <10 nm Surface area: 288 m^2/g Amorphous and crystalline phases: 67.2% Anatase, 32.8% Amorphous	*Pseudokirchneriella subcapitata*	Growth rate (72 h)	–	–	3.3	241	[33]
Anatase. Content of amorphous TiO_2 not specified.			–	–	18.0	145	
Primary particle diameter: ~30 nm Surface area: 47 m^2/g Amorphous and crystalline phases: 72.6% Anatase, 18.4 Rutile, 9% Amorphous			–	–	15.5	71.1	
Primary particle diameter: 21 nm Surface area: 55.13 m^2/g Amorphous and crystalline phases: 72.6% Anatase, 18.4% Rutile, Amorphous: 9%	*Pseudokirchneriella subcapitata*	Growth inhibition (72 h; the sample irradiated before testing)	< 0.5	–	–	Visible: 2.53 UVA: 3.00 UVB: 2.49	[34]
TiO_2-content: 99.7% Primary particle diameter: <25 nm Surface area: 200-220 m^2/g	*Scenedesmus sp.*	Growth inhibition (72 h)	1.2	–	–	21.2	[31]

Characteristics of the tested nano-TiO_2	Test organism	Toxicology endpoint	NOEC	LOEC	EC_{10}	EC_{50}	Reference
Primary particle diameter: ≤ 10 nm	*Tetrahymena thermophila*	growth inhibition (28 h)	no observed effect up to 400 mg L^{-1}				[35]
Primary particle diameter: 25–70 nm	*Daphnia magna*	Mortality (48 h)	EC_{20}: no observed effect in 20000 mg L^{-1}	–	*EC_{20}: no observed effect in ~20000 mg L^{-1}		[17]
TiO_2-content: 73–83% Lattice structure: rutile Particle size: length: 50 nm; width: 10 nm Specific surface area: 100 m^2/g	*Daphnia magna*	Mean cumulative offspring (21 days)	3.0	10	5.02	26.6	[36]
		Mortality (21 days)	30	100	31.5	66.1	
Particle size: diameter: ~6 nm	*Daphnia magna*	Immobilization (48 h)	no observed effect between: 2.5–250 mg L^{-1}				[29]
Primary particle diameter: 6 nm Surface area: 230 m^2/g Zeta-potential: 53.5 mV	*Daphnia magna*	Semi-static reproduction tests- commulative offspring (21 days)	–	0.06	–	–	[37]
		Semi-static reproduction tests- body length (21 days)	–	0.2	–	–	
Primary particle diameter: 21 nm Surface area: 50 m^2/g Zeta-potential: 32.1 mV		Flow through test- commulative offspring (21 days)	no observed effect between: 0.02–2 mg L^{-1}				
		Flow through test- body length (21 days)					
Primary particle diameter: 15 nm Surface area: 240 m^2/g Amorphous and crystalline phases: 100% Anatase	*Daphnia magna*	Immobilization (72 h)	–	–	–	1.3	[38]
Primary particle diameter: 25 nm Surface area: n.a. m^2/g Amorphous and crystalline phases: 100% Anatase			–	–	–	3.15	
Primary particle diameter: 32 nm Surface area: 45 m^2/g Amorphous and crystalline phases: 100% Anatase			–	–	–	3.44	
Primary particle diameter: 21 nm Surface area: 50±15 m^2/g Amorphous and crystalline phases: 81% Anatase, 19% Rutile	*Lemna minor*	Growth inhibition-frond number (14 days, renewal)	no observed effect between: 0.01–5 mg L^{-1}				[39]

–: Data not available

DLS: Median values for particle size in medium determined with dynamic light scattering method

*: If EC_{10} is not available, other EC_x values are given

The mass and the number size distribution were determined at 25°C by using a dynamic light scattering (DLS) device (Malvern Zetasizer ZS, Malvern Instruments, UK) that was operated with a He-Ne laser light at a wavelength of 633 nm. Light scattering was detected at an angle of 173°. Three replicate measurements were made immediately after dispersion in MilliQ® ultrapure water. The characteristics of the applied $nTiO_2$ suspension are shown in Table 2.

Table 2 Characteristics of the applied Degussa VP P90 $nTiO_2$ suspension

Average primary particle size [nm]	14
Specific surface area [m^2/g]	90 ± 20
Mean particle diameter by mass [nm][a]	73
Mean particle diameter by number [nm][b]	58
Concentration [w/w%][c]	4.00
Crystalline phase	90% anatase; 10% rutile

[a] Mean particle diameter by mass : the summarized volume of 50% of particles found in the dispersion is above this value, 50% is under this value. Determined by DLS method.

[b] Mean particle diameter by number: 50% of the particles found in the dispersion is above this size, 50% is under this size. Determined by DLS method.

[c] Mass percentage; 100 g of suspension contains 4 g of $nTiO_2$ by dry weight

A dilution series was prepared from the original stock suspension by diluting with MilliQ® ultrapure water. The preparation of this dilution series with MilliQ® ultrapure water was crucial. Directly diluting the stock suspension with the inorganic salts containing medium should be avoided due to eliminating the possibility of any aggregation or agglomeration phenomena, hence the changing of the concentration of the nano-size particles in the medium. The members of the dilution series were added in appropriate proportion into the test systems to set the exact test concentration in the test medium. The applied nominal concentrations in case of all test organisms are collected in Table 3.

Table 3 Applied $nTiO_2$ nominal concentrations

Test organism	Concentrations [$\mu g\ L^{-1}$]
Aliivibrio fischeri	0.01; 0.1; 1; 10; 100; 1000; 10,000; 100,000
Chlorella vulgaris *Pseudokirchneriella subcapitata* *Scenedesmus subspicatus*	80; 400; 1625; 2000; 3125; 6250; 10,000; 12,500; 25,000; 50,000
Tetrahymena pyriformis	0.1; 1; 10; 100; 1000; 10,000
Daphnia magna	0.05; 0.5; 5;50; 500
Lemna minor	0.1; 1; 10; 100; 1000; 10,000

2.2 Test organism

2.1.1 *Aliivibrio fischeri* bacterium cultures

The applied bacterium strain (NRRL B-111 77) was cultured and maintained under axenic circumstances. The ingredients of the growth medium (pH=7.2): 30 g NaCl, 6.1 g $NaH_2PO_4 \cdot H_2O$, 2.75 g K_2HPO_4, 0.204 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g $(NH_4)_2HPO_4$, 5 g peptone, 0.5 g yeast extract, 3 cm^3 glycerol per 1 L distilled water [40].

2.1.2 Unicellular alga species

The applied alga strains (*Pseudokirchneriella subcapitata, Scenedesmus subspicatus, Chlorella vulgaris*) were cultured and maintained on agar slant cultures in the laboratory. The following algal growth medium was solidified with 2% agar: Macroelement solution (100x dilution) ingredients for 1 L distilled water: NH_4Cl: 1500 mg L^{-1}; $MgCl_2 \cdot 6H_2O$: 1200 mg L^{-1}; $CaCl_2 \cdot 2H_2O$: 1800 mg L^{-1}; $MgSO_4 \cdot 7H_2O$: 1500 mg L^{-1}; KH_2PO_4: 160 mg L^{-1}. Fe-solution (1000x dilution) ingredients for 1 L distilled water: $FeCl_3 \cdot 6H_2O$: 80 mg L^{-1}; $Na_2EDTA \cdot 2H_2O$: 100 mg L^{-1}. Carbonate-solution (100x dilution) ingredients for 1 L distilled water: $NaHCO_3$: 50 mg L^{-1} (non autoclavable). Microelement solution (1000x dilution) ingredients for 1 L distilled water: H_3BO_3: 185 mg L^{-1}; $MnCl_2 \cdot 4H_2O$: 415 mg L^{-1}; $ZnCl_2$: 3 mg L^{-1}; $CoCl_2 \cdot 6H_2O$: 15 mg L^{-1}; $CuCl_2 \cdot 2H_2O$: 0.05 mg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$: 7 mg L^{-1}.

To ensure the validity of the data and sensitivity of unicellular algal species potassium dichromate as reference toxicant was measured twice a year.

2.1.3 *Tetrahymena pyriformis* cultures

Tetrahymena cultures (A-759-b) were maintained in the laboratory in PP medium (1% tryptone, 0.1% yeast extract) with antibiotic mix (0.2% penicillin G sodium, 2% streptomycin sulphate, 1% nystatin) described by Leitgib et al. [40]. Axenic cultures of *T. pyriformis* were grown for 24 h in the dark at 22°C.

2.1.4 *Daphnia magna* cultures

A colony of *Daphnia magna* cultured in the laboratory was used in this experiment. The test animals were cultured in a 5 L volume beaker in a 21.5±1°C thermostatic chamber with 16:8 h light:dark cycle (illumination: Juwel Aquarium, Day-Lite, 15 W, 438 mm lamp, 560 Lumen, 6500 K). For the test adult (about 10 day old) female animals were used, which were fed every two days by an alga suspension cultivated in the laboratory containing *Scenedesmus obtusiusculus*. For maintaining *Daphnia magna* aged, dechlorinated tap water was used. Its electric conductivity value was presumably less than 500 mS cm^{-1} [41].

To check the sensitivity of the *D. magna* culture acute toxicity tests were performed with the potassium dichromate ($K_2Cr_2O_7$) as reference toxicant about every six months. Sensitivity of *D. magna* culture to $K_2Cr_2O_7$ was within the limits (EC_{50}, 24 h = 0.6–2.1 mg L^{-1}) as set by the guideline OECD 202.

2.1.5 *Lemna minor* cultures

A colony of *Lemna minor* cultured in the laboratory was used in this experiment. The test organisms were cultured in a 20x30x7 cm glass container in a 21.5±1°C thermostatic chamber with 16:8 h light:dark cycle (illumination: Juwel Aquarium, Day-Lite, 15 W, 438 mm lamp, 560 Lumen, 6500 K). Two-leaf *L. minor* individuals, cultivated in Hoagland's nutrient medium, were used for the test [42]. 3,5-dichlorophenol was used as a reference chemicals, to check the sensitivity of the *Lemna minor* at least twice a year.

2.3 Test methods and evaluation

2.3.1 *Aliivibrio fischeri* bioluminescence inhibition test

The *Aliivibrio fischeri* bioluminescence inhibition test was carried out by modification of the protocol described by Leitgib et al. [40]. The luminescence intensity was measured with Fluostar Optima microplate reader after 30, 60 and 120 min of contact time. The evaluations of the results were carried out as described by Ujaczki et al. [43]. Copper sulphate was used as standard toxicant in all experiments, to check the sensitivity of the *Aliivibrio fischeri* culture.

2.3.2 Algae growth inhibition test

Alga species applied for the experiment were in logarithmic growth phase. Alga cells were washed off from the agar slant with fresh, sterile alga growth medium. The cell number of the alga inoculum was determined using a Burker chamber and set to 3×10^6 cells/mL. 100 µL $nTiO_2$ suspension was pipetted in the appropriate concentration to wells of a microtiter plate, then 100 µL alga suspension to each well. As a control, distilled water and alga growth medium were used.

The optical density of the members of the $nTiO_2$ suspension dilution was also tested for further correction. During the experiment the plates were continuously illuminated (Juwel Aquarium, Day-Lite, 15W, 438 mm lamp, 560 Lumen, 6500 K; 21.5±1°C).

The optical density of the alga suspension in the wells was measured over a period of five days every 24 h at four different wavelengths (405, 450, 490, 630 nm) by Dialab EL800 spectrophotometer.

2.3.3 *Tetrahymena pyriformis* phagocytic activity test

24 h age *T. pyriformis* culture was prepared for the test by pipetting 5 mL of the culture into 20 mL of sterile medium and 600 µL of antibiotic mix solution, then *Tetrahymena* cultures were incubated in a flat bottom flask at 21±0.5°C in a shaking incubator at 150 rpm for 24 h. The expansion cultures were checked under microscope for healthy morphology and motility. For the phagocytic activity bioassay, 500 µL of *T. pyriformis* cell suspension, 100 µL test solution and 400 µL Chinese ink solution were pipetted into an Eppendorf micro test tube and incubated for 30 min at 21±0.5°C under dark circumstances. The Chinese ink solution was prepared with Losina-Losonsky solution [44] and sterile filtered with 0.02 µm sterile filter. After 30 min of contact time the samples were fixed with 20 µL 1.5% formaldehyde solution. The Chinese ink particle granules formed by phagocytosis (test particles) were counted in 80 cells oculometrically with light microscope (Nikon CH20) under 400x magnification. Each measurement was repeated three times.

2.3.4 *Daphnia magna* heartbeat rate test

The experiment was carried out based on the assay of Villegas-Navarro et al. [45] and Dzialowski et al. [46] with modifications. For the test, 10 day old female non-pregnant animals were used, which did not derive from the first brood and were not fed during the test. Test vessels were kept under the same circumstances as described previously in case of *D. magna* laboratory culture. 10 *Daphnia magna* individuals were placed into 50 mL of the test solutions in a 150 cm^3 volume beaker with the help of a special fabric spoon. As a control, 10 test animals were placed into 50 mL of the culturing medium in a 150 cm^3 volume beaker. After 24 and 48 h contact time the heartbeat rate of the *Daphnia* individuals was measured. The measurement was carried out applying NIKON SMZ800 stereomicroscope. The test animals were placed onto a single cavity microscope slide into a 50–100 µL droplet of the test solution, where the heartbeat rate of each test animal was measured three times for 10 seconds. The electric conductivity value of each sample was less than 500 mS cm^{-1} as recommended by Hebert et al. [41], the dissolved O_2 concentration of each sample was more than 3 mg L^{-1} as recommended by the OECD 202 Guideline [47].

2.3.5 *Lemna minor* growth inhibition test

The experiment was carried out with three parallels in 150 cm^3 beakers as described by Fekete-Kertész et al. [42]. On the first day 10 healthy and two-leaf *L. minor* individuals were placed into 50 mL of each dilution member of the test suspension. Hoagland's nutrient medium was applied as control. The beakers were covered with a translucent plastic film to avoid evaporation and concentration of the test solutions during the experiment. The assembled test systems (beakers) were incubated in a 21.5±1°C thermostatic chamber for 7 days under the following light conditions: 16:8 h light:dark cycle (illumination: Juwel Aquarium, Day-Lite, 15W, 438 mm lamp, 560 Lumen, 6500 K).

On the seventh day *L. minor* individuals were removed from the test solutions, then surface-dried on filter paper to constant weight. The dried biomass was placed into ground-necked test tubes containing 5 mL of 96% ethanol. After 24 hours the optical density of the samples was determined spectrophotometrically (Sanyo SP55 UV/VIS spectrophotometer) at 470, 649 and 664 nm wavelength values. The total chlorophyll content

was determined based on the calculation described by Fekete-Kertész et al. [42].

All toxicity tests used a negative control there no test substance was present. These negative controls served as a quality control in an experiment as well as a reference point. Test samples were compared to these negative controls.

2.4 Statistical analysis

One-way analysis of variance (ANOVA) was performed by STATISTICA 12® software identifying significant effects ($p < 0.05$). Univariate Tests of Significance were performed and the homogeneity of variances was examined. In case of significance, the lowest observed effects concentration value (LOEC) was determined using Dunnett's test ($\alpha = 0.05$).

3 Results

3.1 *Aliivibrio fischeri* bioluminescence inhibition test

The effect of Degussa VP P90 $nTiO_2$ suspension was tested on *Aliivibrio fischeri* bioluminescence intensity. The luminescence intensity was measured with Fluostar Optima microplate reader after 30, 60 and 120 minutes of contact time. Table 4 summarises data on inhibition percentage values. After 30 and 60 minutes contact time only the most concentrated sample showed significant inhibitory effect on the luminescence intensity. After 120 minutes of contact time significant inhibition was found in the concentration range 100–100,000 µg L^{-1}. The lowest observed effect concentration was 100 µg L^{-1} with H%=18.8.

Table 4 Inhibition percentage values after 30, 60 and 120 min contact time determined by *Aliivibrio fischeri* bioluminescence inhibition test

Concentration [µg L^{-1}]	Inhibition [%]		
	30 min	60 min	120 min
0.01	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
0.1	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
1	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
10	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
100	0.0 (0.0–0.0)	0.0 (0.0–0.0)	18.8*(16.6–21.0)
1000	0.0 (0.0–0.0)	0.0 (0.0–0.0)	12.0 (11.4–12.6)
10,000	0.0 (0.0–0.0)	0.0 (0.0–0.0)	29.4*(27.0–31.8)
100,000	59.9*(54.3–65.5)	75.0*(71.6–78.4)	73.8*(71.7–75.9)

*significant inhibition; Average inhibition percentage values with standard deviations.

3.2 Alga growth inhibition test

The effect of Degussa VP P90 $nTiO_2$ suspension on alga growth rate was tested applying three different freshwater alga species (*Pseudokirchneriella subcapitata*, *Scenedesmus subspicatus* and *Chlorella vulgaris*). Each of the three applied alga species showed high sensitivity to $nTiO_2$. In case of *C. vulgaris* 12,500; 25,000 and 50,000 µg L^{-1} concentration resulted in 33, 36 and 49% inhibition, respectively. In case of *P. subcapitata* the applied $nTiO_2$ caused 32–50% inhibition in the 3125–25,000 µg L^{-1} concentration range, while it was slightly toxic in the highest applied concentration (H%=10 in 50,000 µg L^{-1}). In case of *S. subspicatus,* the applied $nTiO_2$ resulted in 20–45 H% in the 3125–50,000 µg L^{-1} concentration range (Fig. 1). Monotonic concentration-dependent adverse effect was experienced only in case of the *C. vulgaris* species.

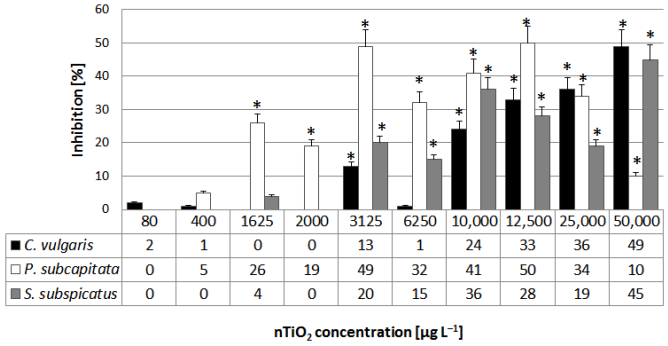

Fig. 1 Effect of the tested $nTiO_2$ suspension on freshwater alga species, given in inhibition percentages (H%). Significant inhibition is marked by asterisk (*).

3.3 *Tetrahymena pyriformis* phagocytic activity test

The modulation of phagocytic activity was investigated as a novel sublethal ecotoxicity measurement endpoint. Figure 2 represents the distribution of the dataset by Gaussian Kernel fitting. The number of test particles formed by phagocytosis is plotted based on the number of observations in 80 cells. As the supplement of Fig. 2, Table 5 collects data on the geometric mean of test particles in the samples.

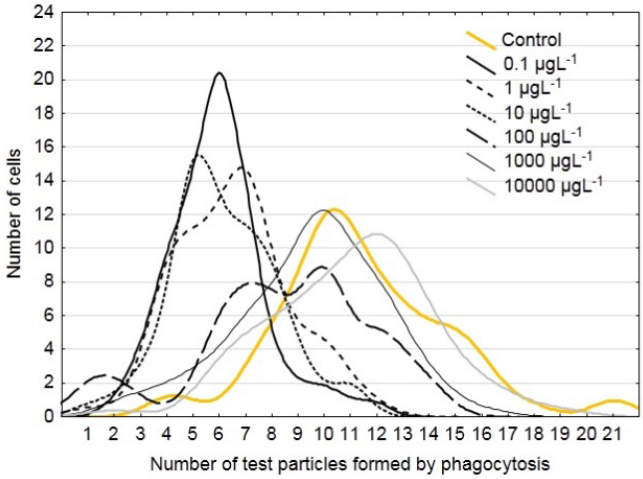

Fig. 2 Distribution of the number of test particles formed by phagocytosis in the dataset by Gaussian Kernel fitting. The results are plotted based on the number of observations in 80 cells.

The applied concentration range was 0.1–10,000 µg L^{-1} in which significant inhibition effect was found within the 0.1–1,000 µg L^{-1} concentration range. We found an inverse relationship between the geometric mean values and concentrations. At the lowest applied concentration *Tetrahymena* could form half the amount of test particles as compared to the control sample. This ratio was nearly the same in case of the 1 and 10 µg L^{-1} samples (Table 5). At the highest concentration of $nTiO_2$ no significant inhibition was experienced.

Table 5 Geometric mean of the test particles formed by phagocytosis in 80 cells.

Concentration [µg L^{-1}]	Geometric mean
Control	11.0
0.1	5.7*
1	6.2*
10	5.8*
100	7.5*
1000	9.0*
10,000	10.5

*significant inhibition

3.4 *Daphnia magna* heartbeat rate test

The adverse effect of Degussa VP P90 $nTiO_2$ suspension was tested on *D. magna* heartbeat rate as an innovative sublethal ecotoxicity endpoint. The applied concentration range was 0.5–500 µg L^{-1} in which significant inhibitory effect was observed in all of the tested concentrations after 24 h of contact time (Fig. 3). Table 6 collects inhibition percentage (H%) data on the effect of Degussa VP P90 $nTiO_2$ on *D. magna* heartbeat rate.

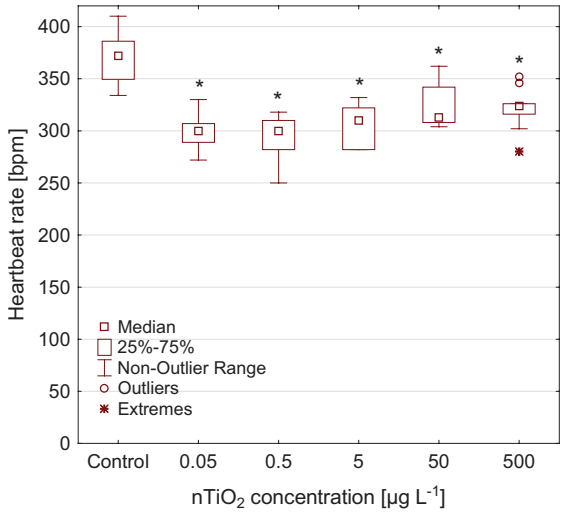

Fig. 3 Box Plot diagram of the effect of the tested $nTiO_2$ suspension on *D. magna* heartbeat rate after 24 h of contact time. Statistically significant decrease in heartbeat rate compared to the control group is marked by asterisks (*). In the diagrams the midpoint represents the mean, the upper and the lower line of the box represent the 25th and 75th percentiles of the distribution, respectively; the whiskers represent the mean ± SD.

Table 6 Inhibition percentage values (H%) of *D. magna* heartbeat rate test after 24 h of contact time.

Concentration [µg L^{-1}]	Inhibition percentage values [H%]
0.05	15*
0.5	17*
5	17*
50	20*
500	20*

*significant inhibition

3.5 *Lemna minor* growth inhibition test

The adverse effect of Degussa VP P90 $nTiO_2$ suspension was investigated on the total chlorophyll content of *L. minor* (Fig. 4). 1–1000 µg L^{-1} concentration $nTiO_2$ resulted in 17–32% inhibition, while in case of higher concentration (10,000 µg L^{-1}) visible aggregation was experienced and there was no significant inhibitory effect on *L. minor* total chlorophyll content (Table 7).

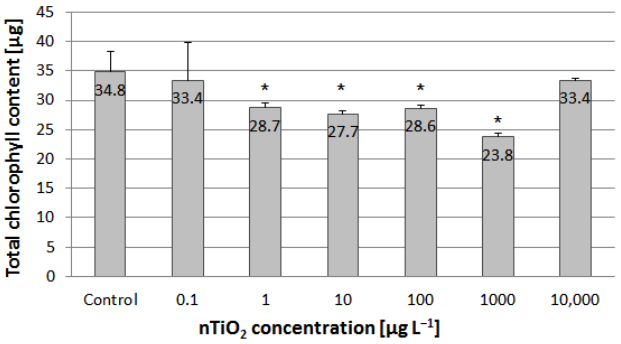

Fig. 4 The effect of $nTiO_2$ on *Lemna minor* total chlorophyll content. Significant decrease of the chlorophyll content is marked by asterisk (*).

Table 7 Inhibition percentage values (H%) of *L. minor* growth inhibition test after 7 days of contact time.

Concentration [µg L^{-1}]	Inhibition percentage values [H%]
0.1	4
1	18*
10	21*
100	18*
1000	32*
10,000	4

*significant inhibition

Lowest observed effect values determined by the applied test organisms at different trophic levels are summarized in Table 8.

Table 8 Lowest observed effect values (LOEC) determined by different test organisms

Test organism	Ecotoxicity endpoint	LOEC [μg L^{-1}]
Aliivibrio fischeri	bioluminescence intensity (120 min)	20
Chlorella vulgaris	growth inhibition (5 d)	3125
Pseudokirchneriella subcapitata	growth inhibition (5 d)	1625
Scenedesmus subspicatus	growth inhibition (5 d)	3125
Tetrahymena pyriformis	phagocytic activity (30 min)	0.1
Daphnia magna	heartbeat rate (24 h)	0.05
Lemna minor	chlorophyll content (7 d)	1

According to the results, the cladocerans *D. magna* and ciliated protozoon *T. pyriformis* demonstrated high acute sensitivity compared to the other test organisms. Compared to the results obtained with *D. magna* and *T. pyriformis,* the alga growth inhibition test applying three different freshwater alga species (*Pseudokirchneriella subcapitata*, *Scenedesmus subspicatus* and *Chlorella vulgaris*) was less sensitive to Degussa VP P90 $nTiO_2$ suspension.

4 Discussion

In our study the ecotoxicity of Degussa VP P90 nano TiO_2 was investigated with test organisms of different trophic levels. A marine bioluminescent bacterium (*Aliivibrio fischeri),* freshwater algae (*Pseudokirchneriella subcapitata, Scenedesmus subspicatus* and *Chlorella vulgaris)*, and a ciliate protozoon *Tetrahymena pyriformis* were applied as unicellular model organisms. The freshwater aquatic macrophyte *Lemna minor* (common duckweed) and a freshwater crustacean *Daphnia magna* (water flea) were used to determine the adverse effects of $nTiO_2$ on higher trophic levels.

Supplementary information about the literature data discussed here, can be found in Table 1 regarding the properties of the applied $nTiO_2$ and the ecotoxicity measurement endpoints.

During our research the *Aliivibrio fischeri* bioluminescence inhibition test showed significant sensitivity (H% = 18.8–73.8) after 120 minutes of contact time in the 100–100,000 μg L^{-1} concentration range. However a few researchers reported that the ~6 nm and 25–70 nm particle size $nTiO_2$ had no inhibitory effect on *A. fischeri* neither at 20,000 mg L^{-1} or 0.4395–112.5 mg L^{-1} concentration range [17, 29]. According to our results the toxic effect depends on duration of exposure. While 20 μg L^{-1} $nTiO_2$ did not show any difference in bioluminescence of *Aliivibrio fischeri* after 30 min and 60 min contact time, significant decrease (~20%) was observed after 120 min exposure.

In case of testing with algae, growth inhibition is a very common ecotoxicity endpoint. Most of the current literature data is acquired from growth inhibition studies. Ji et al. [30] found EC_{30}(6 day)=30 mg L^{-1}, while Sadiq et al. [31] reported EC_{50}(72 h)=16.12 mg L^{-1} based on growth inhibition of *Chlorella vulgaris*. In our study the EC_{50} value was 50 mg L^{-1}.

Warheit et al. [32] studied the effect of two different particle size $nTiO_2$ on *Pseudokirchneriella subcapitata*, one of them with coating. The coated $nTiO_2$ was two times larger in size than the uncoated one, with a very different specific surface area: 38.5 and 5.8 m^2 g^{-1}, respectively. The larger size (380 nm) $nTiO_2$ resulted in EC_{50}(72 h) = 61 mg L^{-1}, the smaller one (140 nm) resulted in EC_{50}(72 h) = 87 mg L^{-1}. Blaise et al. [15] reported the EC_{25}(72 h) = >100 mg L^{-1}, while Aruoja et al. [9] reported EC_{50}(72 h) = 5.38 mg L^{-1} based on the same method as Warheit et al. [32]. Aruoja et al. [9] used 25–70 nm particle size $nTiO_2$, which is much smaller than the size applied by Warheit et al. [32], and it resulted in a lower EC_{50} value (5.38 mg L^{-1}). Hartmann et al. [33] investigated even smaller particle size $nTiO_2$ (10 and 30 nm) that caused EC_{50}(72 h) = 241 and 71 mg L^{-1}, respectively.

In our research the 73 nm particle size $nTiO_2$ caused 50% inhibition at 3.125 and 12.5 mg L^{-1}, but the effect was not concentration-dependent. Based on these results, we can observe that very small and very large $nTiO_2$ particle sizes exert lower inhibition than particles from an intermediate size range (25–70 nm). *Scenedesmus subspicatus* was nearly as sensitive as *C. vulgaris* to the 25 nm size $nTiO_2$ (EC_{50}(72 h) = 21.2 and 16.12 mg L^{-1}), respectively. In our research the 73 nm $nTiO_2$ particle size caused 45% inhibition at 50 mg L^{-1}, but the effect was not concentration-dependent. Authors reported that NPs can be bound on the cell surface of the algal species e.g. *Pseudokirchneriella subcapitata* and *Chlorella sp*. [31, 48]. Large $nTiO_2$ aggregates can entrap algal cells, which may contribute to the inhibition of algae growth [9]. The mode of action of $nTiO_2$ particles to algal cells is still unknown, but they were shown to induce the production of reactive oxygen species, causing cell membrane damage, protein oxidation and possible DNA damage [26, 49, 50].

As seen in Table 1, the particle size, the specific surface area, the crystalline phase composition, and the presence of surface coating are variable factors, which may have strong influence on the toxicity even within the same particle size range.

The modulation of phagocytic activity by $nTiO_2$ was tested with *Tetrahymena pyriformis*. Currently only few data can be found in literature about the effect of $nTiO_2$ on *T. pyriformis*. According to our results, the phagocytic activity bioassay showed outstanding sensitivity to TiO_2 nanoparticles: administration of 0.1 μg L^{-1} $nTiO_2$ resulted in 50% inhibition. Ud-Daula [35] did not experience any inhibition effect of the <10 nm size $nTiO_2$ at up to 400 mg L^{-1} concentration in a 28 h contact time experiment. But there are some reports on decrease in membrane fluidity after exposure of *Tetryhymena* species to titanium dioxid [21]. So our findings can be in associated with alterations in membrane structure.

Our microscopic studies provided exciting information about the fate of $nTiO_2$ particles inside the protozoan cell. Although the protozoan cells are able to ingest $nTiO_2$ particles, they are unable to digest them. The $nTiO_2$ particles in the test medium are ingested immediately and food vacuoles filled with $nTiO_2$ particles are formed. This filling of vacuoles was also detected by Rajapakse research group [21]. In these vacuoles the cell stores the agglomerated $nTiO_2$ particles until they are exocytosed into the test medium as a larger aggregate (Fig. 5). Therefore, *Tetrahymena* can influence the bio-aggregation of nanoparticles in the aquatic environment [35]. Throughout these processes of endocytosis and exocytosis, TiO_2 particles interfere with cell growth and consequently can induce acute toxicity. Because *Tetrahymena* can internalize and accumulate TiO_2 nanoparticles this accumulation may exert harmful effects via a food web transfer by increasing the risks of transfer of these NPs to higher trophic levels in the ecosystem.

At this phase of the research we aimed to study the $nTiO_2$-mediated alterations in feeding behaviour and to assess potential impact on it. Then further studies are necessary to explore the mechanisms of inhibition of feeding behaviour and demonstrate how the $nTiO_2$ concentration and contact time influence the phagocytotic activity.

An innovative sublethal ecotoxicity endpoint, the heartbeat rate, was applied in a 24 h contact time *Daphnia magna* acute ecotoxicity test. The *Daphnia magna* heartbeat rate test presented outstanding sensitivity with significant inhibition percentage values of 15–20% at the applied concentration range 0.5−500 µg L^{-1}. Heinlaan et al. [17] did not experience inhibition effect of the applied 25–70 nm particle size $nTiO_2$ in case of 48 h contact time mortality test even at 20,000 mg L^{-1} concentration. Strigul et al. [29] reported no immobilization effect of a ~6 nm $nTiO_2$ particle size after 48 h in the 2.5–250 mg L^{-1} concentration range, while Clément et al. [38] measured 1.3, 3.15 and 3.44 mg L^{-1} EC_{50}(72 h) values for immobilization when testing with 15, 25 and 32 nm size TiO_2 particles, respectively. Chronic reproduction tests could detect the adverse effect of $nTiO_2$ to *D. magna* in smaller concentration ranges. Seitz et al. [37] determined 0.06 and 0.2 mg L^{-1} as LOEC values in case of cumulative offspring and body length measurement endpoints in 21 days reproduction tests, respectively for the 6 nm particle size $nTiO_2$. However, they did not find any inhibition effect when testing with a 21 nm particle size $nTiO_2$ at the 0.02–2 mg L^{-1} concentration range. Since *D. magna* is a filter feeding organism, it can accumulate a significant amount of TiO_2 NPs from the test environment, which may cause abnormal food intake or defecation, ultimately affecting physiological parameters, growth and reproduction [27].

Both *Tetrahymena sp*. and *Daphnia sp.* are important members of food chain in aquatic environments. Alterations in behaviour and physiology of these organisms can be indicator of larger ecosystem effects.

Our study demonstrated that TiO_2 nanoparticles were accumulated in protozoon and in water flea, consequently they can be transferred to higher trophic level animals with the occurrence of biomagnification.

The adverse effect of nano-scale TiO_2 suspension on the total chlorophyll content of *L. minor* was investigated. We experienced the aggregation and sedimentation of the $nTiO_2$ particles in the 10 mg L^{-1} concentration sample while the total chlorophyll content was not reduced in this sample compared to the control. In our study 0.001–1 mg L^{-1} concentration $nTiO_2$ resulted in 17–32% inhibition, sample compared to the control.

In our study 0.001–1 mg L^{-1} concentration $nTiO_2$ resulted in 17–32% inhibition, while Li et al. [39] reported no inhibition effect in the 0.01–5 mg L^{-1} concentration range, when testing a 21 nm particle size $nTiO_2$. Li et al. [39] found that *L. minor* accumulated nano-TiO_2 by surface accumulation. The accumulation was caused by surface attachment of the particles onto plant cell walls with strong adhesion. Although TiO_2 NPs are

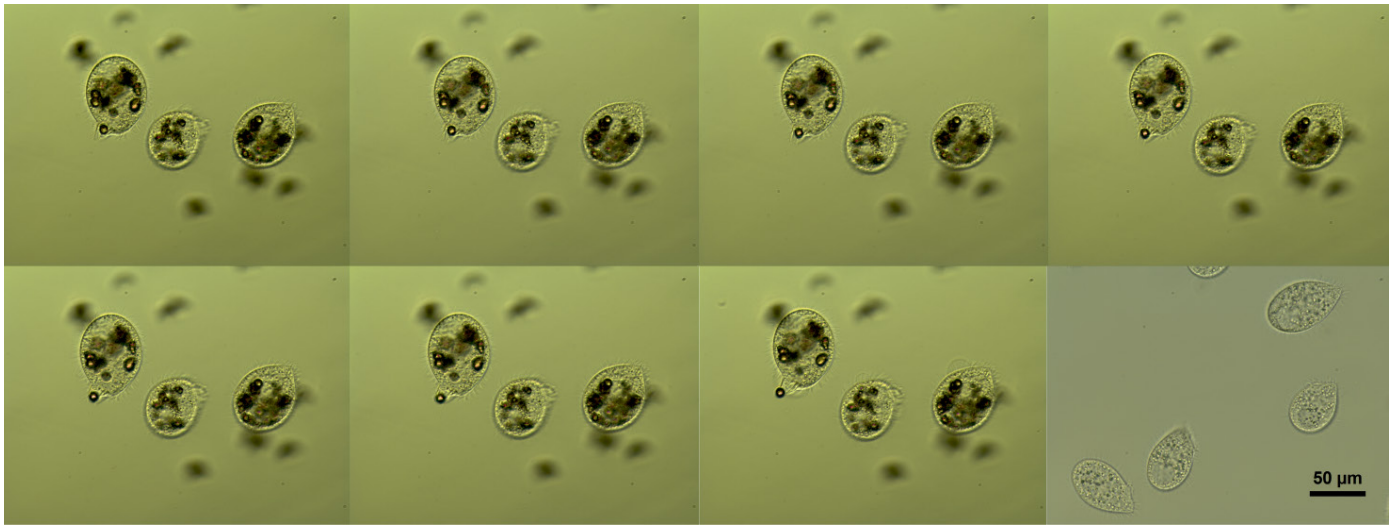

Fig. 5 The process of exocytosis: $nTiO_2$ agglomerates are being exocytosed from the food vacuoles (24 h exposure, 100 µg L^{-1} $nTiO_2$ concentration). The last picture shows the control cells without $nTiO_2$ aggregates, vacuoles were not filled. (NIKON ECLIPSE E400 fluorescence microscope; 600x magnification).

not able to penetrate into the cells of plants and algae, the reactive groups of the plant cell walls give an opportunity to interact with nanoparticles in the test medium [31].

Currently there are only a limited number of data concerning observed environmental concentrations of TiO_2 nanoparticles. In most cases the levels of its removal and the release from waste water treatment plants were measured [25, 49]. The TiO_2 nanoparticle concentrations in sewage treatment plant effluents ranged from 1.37 μg L^{-1} to 220 μg L^{-1} [25, 51].

A few studies modelled the quantities of nanosized TiO_2 released into the surface waters.

Predicted environmental concentrations of TiO_2 nanoparticles in the water compartment in different countries ranged from 0.002 μg L^{-1} to 16 μg L^{-1} [25, 51].

In our study the lowest observed effect concentrations determined by *Tetrahymena pyriformis* phagocytotic activity and *Dahnia magna* heartbeat rate tests were 0.1 μg L^{-1} and 0.05 μg L^{-1}, respectively. These quantified results of behavioural and physiological assays reflect the realistic concentrations that may occur in the ecosystem. Consequences of our study clearly indicate that nanosized TiO_2 may impact the aquatic ecosystem.

5 Conclusion

In the reflection of our results and the current literature data, the ecological consequences of TiO_2 NPs may have a greater importance than previously considered. Since bacteria, protozoa, unicellular algae, daphnids and duckweed are important members of the aquatic food chain and can serve as food to other aquatic organisms increased bioaccumulation can be expected even if results show that the applied TiO_2 NP has no adverse effect on the particular test organism.

This study applying sensitive endpoints clearly indicated the adverse effects of TiO_2 nanoparticles to aquatic life. In addition the study confirms the benefit of behavioural and physiological assays in assessing the impact of nanoparticles and demonstrates the uses of these sublethal endpoints to better understand exposure to nanoparticles at sublethal levels. As the production and the use of nanoparticles increases the exposure and the impact becomes more likely.

There are conflicting opinions about the testing of NPs, whether they should be tested in their stabile form by continuous medium renewal during the test or rather testing the environmentally more relevant aggregated form. Due to the tunable properties of NPs determined by their minute particle size both recommendations should be considered. The mode of action of $nTiO_2$ can vary from test organism to test organism and in most cases the mechanism underlying the interaction is still unknown.

Due to the high variety of TiO_2 NPs, data from different authors can be contradictory even if applying the same test organism and the same test protocol. Therefore extensive data is required to get a comprehensive perspective of $nTiO_2$ ecotoxicity.

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