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Toxicity of dimercaptosuccinate-coated and unfunctionalized magnetic iron oxide nanoparticles towards aquatic organisms†

Ya-Qi Zhang,^a Ralf Dringen,^{bc} Charlotte Petters,^{bc} Wiebke Rastedt,^{bc} Jan Köser,^a Juliane Filser^d and Stefan Stolte^{*ae}

Magnetic iron oxide nanoparticles (IONP) have gained growing attention in recent years for their promising applications in medical treatment and environmental remediation. Among the IONP, dimercaptosuccinic acid coated-IONP (DMSA-IONP) have great potential because of their rapid uptake into cells and their potential to effectively adsorb heavy metals. The widespread use and potential release of IONP into the environment raises concern about their environmental impact. To date, little is known about the consequences of the exposure of aquatic organisms to such particles. In this context we investigated the colloidal stability of DMSA-IONP in different test media as well as their effects on green algae (*Raphidocelis subcapitata*), duckweed (*Lemna minor*) and water fleas (*Daphnia magna*). Moreover, a comparative analysis of stability and ecotoxicity data of DMSA-IONP with freshly prepared and aged uncoated IONP dispersions was performed, considering the importance of stability of particles in determining their toxicity. The green algae were the most sensitive organism with EC₅₀ values (72 h) ranging between 0.86–2.27 μM Fe (*i.e.* 0.05 to 0.13 mg Fe L⁻¹) for the three types of IONP. The observed flocculation and (co-)sedimentation of algae with IONP are assumed to reduce the access of cells to nutrients and light. *Lemna* was not affected by any of the IONP due to the low availability of IONP induced by fast aggregation of IONP in the medium. Minor toxic effects on *Daphnia* were found for uncoated IONP (EC₅₀ between 374–1181 μM Fe, *i.e.* 21–66 mg Fe L⁻¹) after 72 h. However, the ingestion and accumulation of coated and uncoated IONP in the gastrointestinal tract of daphnids was observed. Our evaluation of IONP has revealed a certain hazard potential to aquatic organisms. In this light it appears important to prevent the release of large amounts of IONP into the environment, which might limit their applicability.

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In recent years iron nanoparticle-based technologies have attracted great interest in the field of wastewater treatment and groundwater remediation. From this application it is very likely that such particles are continuously released into aquatic ecosystems. However, little is known on aquatic toxicity of uncoated and DMSA coated IONP. This study investigates the colloidal stability and ecotoxicity of DMSA-IONP, fresh and aged uncoated IONP to three typical aquatic organisms: *Raphidocelis*, *Lemna* and *Daphnia*. In comparison to ferric and ferrous salts all three types of IONP cause much stronger, short-term toxicological effects to green algae *R. subcapitata*. The EC₅₀ values for the three types of IONP ranged between 0.86–2.27 μM Fe (*i.e.* 0.05 to 0.13 mg Fe L⁻¹) whereas the salts at concentrations higher than 1.0 mg Fe L⁻¹ still significantly stimulated algal growth. Thus, IONP do pose a certain risk that needs to be considered when implementing IONP-based wastewater and remediation technologies.

^a UFT - Centre for Environmental Research and Sustainable Technology, Department Sustainable Chemistry, University of Bremen, Leobener Straße, D-28359, Bremen, Germany. E-mail: stefan.stolte@uni-bremen.de

^b CBIB - Centre for Biomolecular Interactions Bremen, Neurobiochemistry, Faculty 2 (Biology/Chemistry), University of Bremen, Leobener Straße/NW2, D-28359, Bremen, Germany

^c UFT - Centre for Environmental Research and Sustainable Technology, Department Neurobiochemistry, University of Bremen, Leobener Straße, D-28359, Bremen, Germany

^d UFT - Centre for Environmental Research and Sustainable Technology, Department General and Theoretical Ecology, Faculty 2 (Biology/Chemistry), University of Bremen, Leobener Straße, D-28359, Bremen, Germany

^e Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

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1. Introduction

In recent decades magnetic iron oxide nanoparticles (IONP), having an iron oxide core of either magnetite (Fe₃O₄) or maghemite (Fe₂O₃), have been intensively developed for a wide range of applications in biomedical and environmental technology, due to not only the high surface area, but also their unique magnetic properties and high catalytic abilities.¹ These applications include biomedical imaging, drug-loaded targeted delivery, and cell tracking in tumor therapy,² as well as soil and groundwater remediation³ and wastewater treatment.⁴ IONP have been reported as a highly efficient, cost-



effective and convenient³ adsorbent in the removal of toxic metals from aqueous solutions by adsorption. Nassar found that the adsorption capacity of Fe₃O₄ NP for Pb(II) ions was 36.0 mg g⁻¹, which is much higher than that reported for other adsorbents.⁵ Adsorption of Cr(VI) by γ -Fe₂O₃ was also successful, so that the effect of adsorbate concentration and competing ions in solutions could be ignored.⁶ Metals can then be readily separated and removed by applying external magnetic fields.⁷ However, uncoated IONP are susceptible to air oxidation⁸ and tend to aggregate in solution⁴ in inhospitable conditions. The reactivity, mobility⁴ and magnetism¹ can subsequently disappear. As a consequence, IONP are often coated with organic or inorganic compounds, such as polymers,^{2,9} gold,¹⁰ dextran¹¹ or humic acid,¹² not only to improve their colloidal stability and eventually protect IONP from oxidation in aqueous media, but also to equip IONP with unique and specific surface functionalities for cell labeling and targeting, as well as for ion binding to enhance the capacity for heavy metal adsorption in water treatment procedures.⁴

Among the functionalized IONP, those coated with *meso*-2,3-dimercaptosuccinic acid (DMSA, Fig. 1A) present great potential in both target-drug delivery¹³ and remediation of heavy metal pollution.¹⁴ DMSA, a dithiol, is a derivative and an analogue of dimercaprol that contains two sulfhydryl groups (-SH).^{15,16} Oxidation of DMSA molecules around IONP generates a cage of disulfide-cross-linked DMSA molecules around the IONP core, which establishes a negative surface charge of the particles due to an excess of carboxylate groups¹⁷ (Fig. 1B).

The ability of sulfhydryl-containing compounds to chelate metals, *e.g.* lead, mercury, arsenic, and cadmium, has been well studied.^{15,16,18} DMSA is not toxic to humans and has been approved for clinical use for chelation therapy.¹⁹ Accordingly, DMSA can be used as a treatment for arsenic^{18,20} and lead intoxications^{18,21} *in vivo* and heavy metal removal in the environment¹⁴ due to its effectiveness in metal chelation.^{15,18} Moreover, it shows no toxicity in the treatment of heavy metal intoxications²² such as arsenic in mice and rabbits,^{23,24} and can be orally administered without causing redistribution of metals from one organ to another.^{23–25}

In addition, DMSA-IONP have a high capacity and selectivity for heavy metals.¹⁴ The thiol group on the surface of IONP mainly reacts with heavy metal ions directly to form stable

metal-sulfur complexes through chelation.²⁶ In terms of the adsorption coefficient (K_d) values, DMSA-coated Fe₃O₄ IONP are 3–40 times superior to commercial sorbents such as Duolite GT-73 resins and activated carbon Darco KB-B.¹⁴ Moreover, the addition of affinity ligands to the surface of the nanoparticles increases the affinity of the sorbents²⁷ to a wide range of heavy metals with specificity. Compared to uncoated Fe₃O₄ NP, enhancement of heavy metal ions (*e.g.* Hg²⁺, Ag⁺, Pb²⁺, and Cu²⁺) adsorption for DMSA-coated Fe₃O₄ NP was also observed.¹⁴ These ions effectively bind to the DMSA ligands or the iron oxide lattices, and could be separated from solution within a minute with a 1.2 T magnet.¹⁴ Singh *et al.* also found a strong affinity of DMSA-coated Fe₃O₄ NP to Cr³⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, Pb²⁺ and As³⁺ present in wastewater.²⁶

Although a number of laboratory studies and field trials have indicated IONP-based technology as a promising tool for heavy metal removal in wastewater treatment due to the advantages, such as low cost, strong adsorption capacity, easy separation and enhanced stability,⁴ it is still at a relatively early stage for wide application.^{4,7} Many important issues concerning the impact of IONP application on human health and environment remain poorly understood, which may significantly limit the widespread application of IONP as remediation tools.^{3,7} So far, most studies have focused on the effect of IONP on mammal cells. Related studies have estimated the genotoxicity and cytotoxicity of DMSA-IONP (γ -Fe₂O₃), such as to cultured brain astrocytes²⁸ and human dermal fibroblasts,²⁹ but weak or no effects were observed in the highest tested concentrations of 4000 and 1791 μ M Fe (*i.e.* 0.22 and 0.10 g Fe L⁻¹), respectively. While uncoated Fe₂O₃ IONP of 21 nm induced oxidative stress and morphological damage in brain nerve cells of mice when 130 μ g of IONP was instilled into mice.³⁰ On the other hand, very few studies have investigated the effects of IONP, particularly in aquatic environments.³¹ Some data on the effects of IONP on aquatic organisms such as zebrafish (*Danio rerio*,³¹ α -Fe₂O₃) and water flea (*Daphnia magna*,^{9,32} Fe₃O₄) are available but still too few to make comprehensive conclusions, and nothing is known on the effects of DMSA-IONP on aquatic organisms.

In view of the wide range of potential applications of uncoated IONP and particularly of DMSA-IONP it appears very likely that such particles are continuously released into aquatic ecosystems. For example, this could happen through industrial/domestic effluents or discharge of wastewater treatment plants. Therefore, uncertainties about these particles in the aquatic environment need to be investigated and addressed before their widespread application.

In order to anticipate and assess the hazards that DMSA-coated and uncoated IONP might pose in the aquatic environment (Fig. 2), we synthesized magnetic IONP (γ -Fe₂O₃) and (i) evaluated the stability of DMSA-IONP and compared it to fresh and aged uncoated IONP in different aquatic media; (ii) estimated and compared the potential acute/subchronic toxicity of the IONP with three typical model organisms from freshwater: chlorophyte algae (*Raphidocelis subcapitata*),

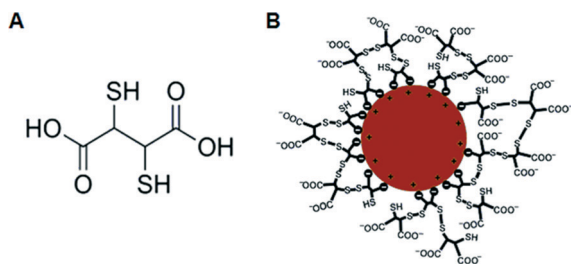


Fig. 1 A. Structural formula of *meso*-2,3-dimercaptosuccinic acid (DMSA); B. DMSA-IONP: DMSA molecules bind to IONP surface via their carboxylate groups and form a cage by disulfide bridges.¹⁷ The thiol groups on the surface can effectively capture heavy metal ions to form strong metal-sulfur complexes.



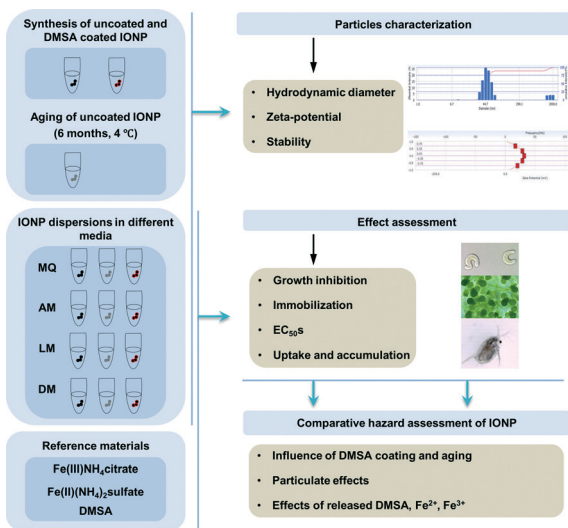


Fig. 2 Outline of the study flow. With MQ = MilliQ water, AM = algae medium, LM = *Lemna* medium, and DM = *Daphnia* medium.

duckweed (*Lemna minor*), and water flea (*Daphnia magna*); (iii) aimed to discriminate between the potential effect of IONP-derived iron ions and IONP in particulate form, considering the effects that released iron ions cause.

2. Materials and methods

2.1 Materials

All chemicals were purchased in the highest purity available from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany). Nunc EASY flasks with 25 and 75 cm² were obtained from VWR (Hannover, Germany). The 6-well plates were standard polystyrene cell culture plates with lids from Greiner Bio-one, Frickenhausen, Germany, and the 24-well tissue culture plates with lids were from Sarstedt, Nümbrecht, Germany. The test kit for immobility/mortality tests of *Daphnia magna* was Daphtoxkit F *magna*, MicroBioTests. Glass containers of 20 mL for colloidal stability testing were neo-Lab EPA thread vials (clear glass ND24, Heidelberg, Germany). Syringe filters (Multoclear, 0.45 μm, RC, 13 mm) for medium filtration were purchased from CS Chromatographie Service (Langerwehe, Germany). MilliQ water (18.2 MΩ cm⁻¹ in resistivity) was produced from Millipore MilliQ Plus water purification system (Massachusetts, USA). All deionized water used in this study, if not specifically stated, was previously filtered by Carbonit NFP Premium-9 water filter (Heidenheim, Germany) with pore size 0.45 μm.

2.2 Organisms and cultures

2.2.1 *Raphidocelis subcapitata*. The cultures of *R. subcapitata* (formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*; strain 61.81) were ordered from the culture collection of algae (SAG), University of Göttingen, Germany. The stock cultures were kept on agar in glass tubes under natural light at room temperature. Algae

for ecotoxicity tests were first taken from the stock cultures and inoculated in 50 mL OECD TG 201 medium³³ in cell culture flasks (EASY flask, 75 cm²) for a synchronized growth for 11 days in a climate chamber (Thermostatschrank ET 618-4/135) at 22 ± 1 °C and with a light:dark cycle of 14:10 h. The cultures were diluted and checked for cell counts every 3–4 days in order to keep them in exponential growth. Certain amounts of the synchronized cultures were finally diluted in 50 mL medium to reach a count of 2.5 × 10⁴ cells per mL 4 days before the tests. The pH of the medium was adjusted to 8.1 ± 0.2. The flasks were placed on a shaker (IKA®MTS 2/4 digital) at 150 rpm in the chamber. Cultures were illuminated (Sylvania Luxline plus F 15w/865 Daylight de Luxe) at approximate 6600 lux (measured by Panlux Gossen 8 A94256).

2.2.2 *Lemna minor*. The strains of *L. minor* were perennial cultures (originally ordered from Department of Agricultural Science, the Royal Veterinary and Agricultural University (KVL), Taastrup, Denmark) on agar in our research group. Stock cultures of *Lemna* were grown in Erlenmeyer flasks in sterilized Steinberg medium³⁴ (pH 5.5 ± 0.2) in a climate chamber with a constant temperature of 24 ± 2 °C in accordance to OECD guideline 221.³⁴ The chamber was illuminated continuously with a maximum of 6000 lux. Ten colonies with 3 fronds each for ecotoxicity tests were transferred into 150 mL fresh medium 7 days before the tests. Within 2 days before the tests, medium was replaced by fresh medium.

2.2.3 *Daphnia magna*. The ephippia of the *D. magna*, medium components (according to ISO 6341, 67.75 mg L⁻¹ NaHCO₃, 294 mg L⁻¹ CaCl₂·2H₂O, 123.25 mg L⁻¹ MgSO₄·7H₂O, 5.75 mg L⁻¹ KCl) and food (*microalgae Spirulina*) were all contained in the test kit Daphtoxkit F *magna*.³⁵ The medium was prepared by mixing the four components with filtered-sterilized (0.45 μm) deionized water, and the pH was adjusted to 7.0 ± 0.2. The ephippia hatching lasted 85 h at room temperature under continuous illumination of around 6000 lux. The neonates were pre-fed with one vial (recommended by the test kit) of dispersion of the *microalgae* 2 h before exposure.

2.3 Synthetic procedures of IONP

2.3.1 Synthesis of uncoated IONP. Magnetic IONP (γ-Fe₂O₃) were synthesized according to a previously described method.^{36,37} A solution of 380 mL containing 8.89 g (32.9 mM) FeCl₃·6H₂O, 3.28 g (16.5 mM) FeCl₂·4H₂O and 0.75 mL 37% HCl was thoroughly mixed. Precipitation was induced by slowly adding 25 mL 25% (w/v) NH₄OH solution under vigorous stirring. The resulting black magnetic precipitate was collected and isolated by the aid of a permanent magnet (NdFB-magnet), and washed twice with 100 mL deionized water. The precipitate was then heated with 40 mL 2 M HNO₃ until the color of the mixture changed to dark brown. The product was again magnetically collected and separated from the supernatant, and then heated with 60 mL 0.34 M Fe(NO₃)₃·9 H₂O at 90 °C for 30 min. After magnetic separation from the supernatant, the IONP were dispersed with deionized water



to a final volume of 50 mL and then sterile filtered through a 0.2 μm filter (syringe filter, cellulose acetate membrane with pore size 0.2 μm , Sigma-Aldrich Steinheim, Germany).

2.3.2 Iron content determination. The iron content was determined by a modification of the colorimetric ferrozine-based method previously described.³⁷ A volume of synthesized IONP dispersion of 10 μL was mixed with 40 μL 37% HCl followed by dilution with 950 μL 50 mM NaOH; 100 μL of the solution was then mixed with 10 mM HCl to a volume of 200 μL . The sample was mixed with 100 μL freshly prepared iron-releasing reagent (1:1 mixture of 1.4 M HCl and 4.5% w/v KMnO_4 in double-distilled water), and then 30 μL fresh iron-detection reagent (2.5 M ammonium acetate, 1 M ascorbate, 6.5 mM ferrozine and 6.5 mM neocuproine) was added.³⁸ After 30 min reaction at room temperature, 280 μL of the sample was filled in wells of a microtiter plate (Sarstedt, Nümbrecht, Germany), and the absorbance at 540 nm of the iron-ferrozine complex was recorded by a Sunrise RC microtiter plate photometer (Tecan, Crailsheim, Germany). Iron content was finally determined by comparing the absorbance of the samples to defined iron standard solutions (FeCl_3 in 10 mM HCl).

2.3.3 Synthesis of DMSA-IONP. After determination of the iron content of the synthesized product, the IONP were coated with DMSA. For this 0.13 g (0.7 mM) of DMSA were dissolved in 150 mL double-distilled water by stirring at 50 $^\circ\text{C}$ and then added to 100 mL 40 mM iron in form of IONP during vigorous stirring. After 30 min at room temperature, the particulate content was separated by centrifugation at 800 g for 5 min. After the removal of the supernatant the particles were resuspended in 80 mL double-distilled water. The pH of the dispersion was adjusted to 10 with NaOH and then to 7.4 with HCl. The resulting dispersion was finally filtered through a 0.2 μm filter. The concentration of the final DMSA-IONP dispersion contained 2.55 g Fe L^{-1} (45.6 mM Fe).

The aged uncoated IONP used in this study were synthesized by the same method but were produced six months before ecotoxicity testing and stored at 4 $^\circ\text{C}$.

2.4 Particle characterization and colloidal stability tests

2.4.1 Dynamic light scattering (DLS). DLS was used to measure hydrodynamic diameters using a Beckman-Coulter DelsaNanoC particle analyzer (Beckman Coulter, Krefeld, Germany). This device features a diode laser (30 mW, $\lambda_0 = 658$ nm) and is able to measure the scattered light at scattering angles of 15 $^\circ$ and 165 $^\circ$. The experiments were carried out at the backscattering angle 165 $^\circ$. The scattered light is detected using a photo multiplier tube and analyzed with a digital correlator. After shaking manually for 10 s to ensure sampling of all particles, a sample volume of 2.5 mL was directly filled in Sarstedt fluorescence cuvettes (polystyrene, $d = 1$ cm, Sarstedt, Nümbrecht, Germany) and was thermostated at 25 $^\circ\text{C}$ in the device for 5 min before the measurement time of 120 s per repetition. For each sample 5 measurements were recorded. For the evaluation of the correlation functions $g^{(2)}$ the properties of pure water for the refractive indices n (658

nm, 25 $^\circ\text{C}$) = 1.3328 and for the viscosity η (25 $^\circ\text{C}$) = 0.8878 cP were used as given by the Beckman Coulter Software. The cumulants method was used to calculate the z-average of the hydrodynamic diameter d and the polydispersity index (PI). Each sample was measured in 3 independent repetitions.

2.4.2 Zeta-potential measurements. Measurements were done by electrophoretic light scattering (ELS) also using the Beckman-Coulter DelsaNanoC. After shaking manually for 10 s to ensure sampling of all particles, a sample volume of 5 mL was directly filled in a Flow Cell and equilibrated with the same conditions mentioned in the DLS section. Here the measurement was done at a scattering angle of 15 $^\circ$. The measurement time was 300 s per repetition. For the measurement evaluation the Smoluchowski equation was used. The refractive index, the viscosity, and the dielectric constant ($\epsilon = 78.3$) of pure water was used as given by the Beckman Coulter software (see DLS). For each sample 3 measurements were recorded. Three independent repetitions for each sample were prepared.

2.4.3 Colloidal stability tests. Stability of DMSA- and uncoated IONP was checked by storing volumes of 10 or 20 mL IONP (1791 and 447.7 $\mu\text{M Fe}$, *i.e.* 100 and 25 mg Fe L^{-1}) in MilliQ water, algae, *Daphnia* or *Lemna* medium in 20 mL glass vials in the dark and avoiding any later movement. Test media were filtered by syringe filter (0.45 μm) before analysis. The same treatment was used for investigating the pH effect on colloidal stability. Therefore 25 mg Fe L^{-1} of IONP was prepared in MilliQ water and adjusted to pH values of the different test media (5.5, 7.0 and 8.1) by addition of 0.01 M NaOH or 0.001 M HCl. Stability was documented by taking photographs (Panasonic DMS-FZ50 and Kaiser RB 260 photo lighting device) of the samples as well as by performing DLS and zeta-potential measurements directly (day 0, t_0) or 1, 3 and 7 days after dispersion in the different media.

2.5 Toxicity tests

2.5.1 Growth inhibition of *R. subcapitata*. *R. subcapitata* in exponential growth phase were taken for the toxicity tests. The medium was OECD TG 201 medium, and the tests were performed based on the OECD 201 guideline,³³ but slightly modified that a light:dark cycle of 14:10 h was used instead of a continuous light. The test set-up was validated according to the guideline using the reference chemical 3,5-dichlorophenol. Algae were exposed to DMSA-IONP, fresh and aged uncoated IONP, iron salts ferric ammonium citrate (FAC) or ferrous ammonium sulfate (FAS) in a concentration range of 0.18–1791 $\mu\text{M Fe}$ (0.01–100 mg Fe L^{-1}), as well as to pure DMSA between 0.00326 to 32.6 mg L^{-1} as a reference. Dispersions were prepared in OECD TG 201 (medium containing 0.24 $\mu\text{M Fe}$) or iron-free medium (OECD TG 201 but prepared without iron and EDTA). The iron-free medium was prepared in MilliQ water, and the algae were washed with this medium 3 times (3000 rpm, *i.e.* 1800 g, 10 min; HeraeusTM LabofugeTM 400R centrifuge) before exposure. All glass containers for this treatment were previously rinsed with 10% HNO_3 3 times. The vessels for ecotoxicity tests were



cell culture EASY flasks of 25 cm², and working volume for the tests was chosen as 20 mL. Algae cell counts at the start of the incubation were 2.5×10^4 cells per mL. Samples were set on shakers at 150 rpm in the same climate chamber with the same conditions as the culturing for 72 h. Cell counts after 72 h in IONP tests were recorded by a cell counting chamber (Neubauer-improved, depth 0.100 mm, 0.0025 mm²) and light microscope (Zeiss, Germany); while in tests for other non-IONP substances the cell counts were recorded by a cell counter and analyzer system (CASY® Model TTC). Cell morphology was also visually analyzed under a light microscope (Olympus BX60 with Sony DXC-9100P 3CCD color video camera). Results on unimpaired growth are expressed as percentage of cell number counts compared to the controls (absence of test substances). Substances were tested in 3 replicates with 6 controls (pure OECD TG 201 medium or iron-free medium) for each test and each test was performed 2 or 4 times to ensure reproducibility of the data.

2.5.2 Growth inhibition of *L. minor*. The test was performed according to a modified version of the test protocol described in Drost *et al.*, 2007.³⁹ Steinberg medium, used for preparing solutions, was prepared as given in OECD TG 221.³⁴ One colony with 3 fronds was exposed to 10 mL DMSA-IONP, fresh or aged uncoated IONP (0.18–1791 μM Fe, *i.e.* 0.01–100 mg Fe L⁻¹) in wells of 6-well cell culture plates for 7 days (culture conditions were the same as for the stock culture). Substances were tested in 4 replicates with 12 controls (pure Steinberg medium) for each test, and each test was performed 4 times. The growth was determined based on the frond area (mm²) recorded by a Scanalyzer from Lemnatec (Würselen, Germany). Results on unimpaired growth rate in the presence of test substances are expressed as percentage of growth rate compared to the controls (absence of test substances).

2.5.3 Acute immobilization of *D. magna*. The ephippia hatching and medium preparation were recommended by the corresponding standard operational procedure of the test Kit Daphtoxkit F *magna*,³⁵ just as cultivation. After 3 days of hatching, the neonates were taken for ecotoxicity tests. The testing procedure of the test Kit was miniaturized modified based on Baumann *et al.* 2014.⁴⁰ In brief, dispersion of DMSA-IONP, fresh and aged uncoated IONP with concentrations of 0.18–1791 μM Fe (0.01–100 mg Fe L⁻¹) were prepared, and 2 mL was filled in each well of 24-well plates. One neonate was placed into each well, and exposure was prolonged to 72 h. Tests were performed at 20–22 °C in darkness. The test set-up was validated according to the guideline using the reference chemical K₂Cr₂O₇. Substances were tested in 10 replicates with 10 controls (pure ISO 6341 medium) for each test. Abnormal behavior (swimming slowly without escape response) of the neonates was recorded visually every 24 h. Results on immobilization are expressed as percentage of not affected organisms compared to the controls (absence of test substances). IONP accumulation in *Daphnia* was observed by stereo zoom microscope (Olympus SZX12 with Olympus DF Plapo 1× PF microscope lens).

2.6 Calculation of iron species

The equilibrium iron species present in algae medium (OECD TG 201) were calculated using the software PHREEQCI (v.3)⁴¹ using the database file minteq.v4. The addition of 10 μM Fe as ferric ammonium citrate ((NH₄)₃Fe(C₆H₄O₇)₂·2H₂O, FAC, Fe³⁺) and as ferrous ammonium sulfate ((NH₄)₂Fe(SO₄)₂·6H₂O, FAS, Fe²⁺) to algae standard medium and iron-free algae medium (OECD TG 201 medium prepared without iron and EDTA) were considered in the speciation calculations. Minor modification of the database file for the calculations was done to uncouple ammonium from redox reactions with nitrite and nitrate by the introduction of the variable Amm as replacement for NH₃ in the database. The components of the medium according to the OECD TG 201 and the pH of 8.1 served as input parameters for the calculations. The chloride content served as charge balance in the calculations. The atmospheric partial pressures of CO₂ (with saturation index SI -3.41) and O₂ (SI -0.678) were used as equilibrium phases in the calculations. As equilibrium phase for iron precipitation ferrihydrite (Fe(OH)₃) was chosen, because the SI of this iron phase was the closest to zero in the media and therefore the most realistic precipitation process in the short timeframe of the experiments.⁴²

Four scenarios were considered to discuss the iron speciation in the media: a) absence of O₂ and no precipitation allowed; b) presence of O₂ (8.3 mg L⁻¹, 0.26 mM) and no precipitation allowed; c) absence of O₂ and precipitation allowed for ferrihydrite; d) presence of O₂ (8.3 mg L⁻¹, 0.26 mM) and precipitation allowed for ferrihydrite.

2.7 Statistical analysis

If not stated otherwise, the data are presented as means ± SD of values from at least two independent experiments with at least three replicates in each. Significance of difference between two groups of data was analyzed by the paired *t*-test using the software R (version 3.1.1) (<https://www.r-project.org/>). *p* > 0.05 was considered as not significant. Dose-response curve and EC₅₀ value estimation for each substance was performed by linlogit or probit model of the 'drfit' package in R.

3. Results and discussion

3.1 Characterization of IONP

The DLS measurements revealed a hydrodynamic diameter (*d*) of 50.4 nm for DMSA-IONP in the diluted dispersions (Table 1). A hydrodynamic diameter of 33.3 nm was

Table 1 Hydrodynamic diameter (*d*) and zeta-potential (*z*) of uncoated IONP and DMSA-IONP dispersed in MilliQ water (1791 μM Fe, *i.e.* 100 mg Fe L⁻¹)

IONP type	<i>d</i> (<i>z</i> -average)		<i>z</i> [mV]	pH
	[nm]	PI		
Fresh uncoated IONP	33.3 ± 1.1	0.19 ± 0.05	41.0 ± 8.2	3.5
Aged uncoated IONP	90.2 ± 1.4	0.32 ± 0.01	36.6 ± 1.2	3.2
DMSA-IONP	50.4 ± 0.3	0.25 ± 0.03	-45.7 ± 0.4	6.8



determined for fresh uncoated IONP, whereas a higher value of 90.2 nm for aged uncoated IONP was observed (Table 1), indicating a small degree of aggregation. Positive zeta-potential values (ζ , 41.0 and 36.6 mV, respectively) of fresh and aged uncoated IONP in MilliQ water were measured, while DMSA-IONP had a negative surface charge as demonstrated by a zeta-potential of -45.7 mV (Table 1). The negative charge of DMSA-IONP was expected due to negatively charged carboxyl groups of the bonded DMSA molecules on the surface of the IONP.¹⁷ Polydispersity indices (PI), a measure of the width of the particle size distribution, ranged from 0.19 to 0.32, indicating an increasing variability in the particle size.

3.2 Colloidal stability of IONP

The colloidal stability of DMSA- and uncoated IONP in MilliQ water as well as in the three test media was investigated by DLS and zeta-potential measurements and additionally documented by photographs (Table 2) over a time period of 24 h. As the fresh and aged uncoated IONP behaved similarly in the test media only data for the fresh IONP are presented.

During this period, uncoated IONP were stable in MilliQ water (MQ), but precipitated rapidly when brought into the other test media (Table 2), indicating their low colloidal stability in the presence of complex media compositions. In comparison to MilliQ water, the hydrodynamic diameters of uncoated IONP were much larger in all types of media (>5000 nm, t_0) (Fig. 3A). Their zeta-potentials were clearly decreased and ranged between $+10$ to 0 mV in the media for algae (AM), *Daphnia* (DM) and *Lemna* (LM) at the beginning of the test (t_0) (Fig. 3B). Similar precipitation was also observed by Zhu *et al.*³¹ when Fe_2O_3 NP were added to zebra fish culture medium (consisting of 64.75 mg L^{-1} NaHCO_3 , 5.75 mg

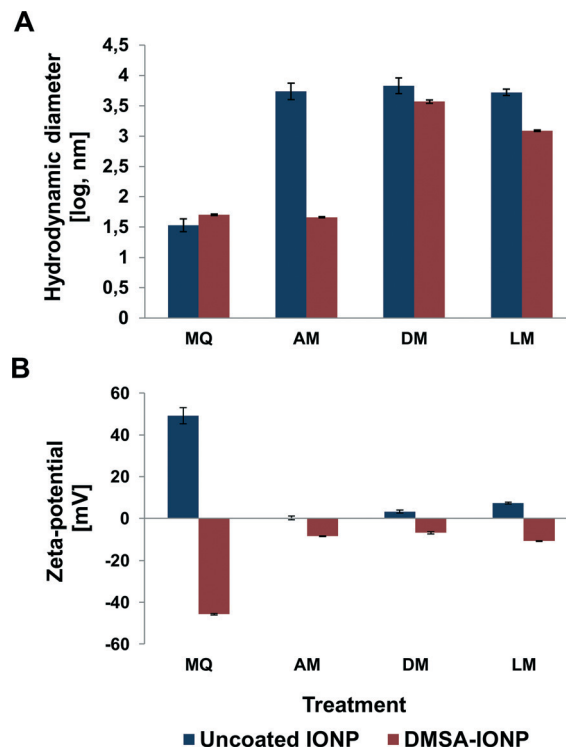


Fig. 3 Hydrodynamic diameter (A) and zeta-potential (B) of DMSA-IONP and fresh uncoated IONP ($1791 \mu\text{M Fe}$, i.e. 100 mg Fe L^{-1}) dispersed in MilliQ water (MQ), algae (AM), *Daphnia* (DM) or *Lemna* (LM) medium at beginning of test (t_0). Data represent mean values \pm SD ($n = 15$ or 9).

L^{-1} KCl, 123.25 mg L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 294 mg L^{-1} $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). The average size of the Fe_2O_3 NP aggregates increased to more than 1 mm from a primary size of 30 nm as measured by DLS.³¹

DMSA-IONP, however, did not precipitate in MilliQ water and algae medium, but in *Lemna* and *Daphnia* media (Table 2). Besides, the precipitation was generally faster in DM than in LM (Table 2). In AM the hydrodynamic diameter was similar to the MilliQ, but much larger in LM ($\approx 1000 \text{ nm}$) and DM ($\approx 3000 \text{ nm}$) (Fig. 3A). The hydrodynamic diameter of dextran-IONP and PVP-IONP (Fe_3O_4) was reported to have increased from 32 nm and 23 nm in pure water to 52.3 and 82.3 nm in *Daphnia* medium (Elendt M7, OECD 202 standard medium), respectively.³² In contrast, the hydrodynamic diameter of ascorbate-IONP and citrate-IONP was still very small (17.5 and 17.2 nm respectively) after being transferred into Elendt M7 medium for 3 days.³²

The zeta-potential of the DMSA-IONP was less negative in AM ($\approx -10 \text{ mV}$) in comparison to MilliQ water ($\approx -46 \text{ mV}$) (Fig. 3B). In DM and LM, particles also appeared less negative (-6 and -19 mV) at the beginning of the test (t_0) but precipitated within 2 h (Table 2) and no zeta-potential could be determined by ELS afterwards. In the study of Baumann *et al.*,³² however, zeta potentials of citrate-IONP were measured as -25 mV in Elendt M7 medium after 5 days that were nearly equal to the one in water which was -27 mV ; citrate-

Table 2 DMSA-IONP and fresh uncoated IONP dispersions ($1791 \mu\text{M Fe}$, i.e. 100 mg Fe L^{-1}) in MilliQ water and the test media

	Time point	MQ	AM	DM	LM
DMSA-IONP	2 h				
	5 h				
	24 h				
Uncoated IONP	5 min				
	1 h				
	5 h				



and PVP-IONP solutions had stayed transparent without visual agglomeration in Elenndt M7 medium after one year.³² In our study, the hydrodynamic diameters (≈ 46 and 49 nm), polydispersity indices (≈ 0.26) and zeta-potentials (≈ -10 and -44 mV) of DMSA-IONP dispersed in AM or MilliQ water, remained almost constant over a period of up to 7 days (ESI† Fig. S1 and S2), and stayed transparent for one year.

To address the concentration depended colloidal stability test at 25 mg Fe L^{-1} were performed (ESI† Tables S1 and S2). Similar behaviors compared to IONP at 100 mg Fe L^{-1} for both types of IONP were observed.

Evidently, DMSA-IONP are colloiddally more stable than uncoated IONP in the three types of test media. The ionized and cross-linked DMSA molecules on the surface provide IONP with not only electrostatic stabilization through high negative charges due to the ionized carboxylate groups,^{13,17} but also steric stabilization due to DMSA polymers.

The decreased surface charges and colloidal stability of uncoated IONP (in the three media) and DMSA-IONP (decreased surface charges in the three media, but decreased stability only in the *Daphnia* and *Lemna* media) compared to MilliQ water may be due to the presence of complex ions and ionic strength (IS) in the media (Table 3). The IS has a strong influence on the surface charge and stability of NP dispersion according to DLVO (Derjaguin–Landau–Verwey–Overbeek) theory.⁴³ High ionic strength leads to aggregation of cobalt ferrite⁴³ and TiO_2 ⁴⁴ nanoparticles by compressing the electrical double layer and decreasing the effective surface potential of particles through formation of a counter ion layer,^{43,44} thus lowering the repulsive barrier magnitude among particles. In particular, divalent cations such as Ca^{2+} and Mg^{2+} have strong effects on bridging between two negatively charged nanoparticles,⁴³ thus aggregating nanoparticles and/or compacting the surface coatings by charge neutralization of the functional groups on NP surface.⁴³ These two cations, when present in concentrations higher than 1 mM , are known to destabilize coated or uncoated NP,⁴³ such as citrate-coated AgNP^{45,46} and silica NP.⁴⁷ Accordingly the im-

mediate aggregation of IONP in LM and DM can be explained: the concentrations of divalent cations in the *Lemna* and *Daphnia* media are around 7 and 10 times higher compared to algae medium (Table 3).

Apart of ionic strength the pH value is known to have a strong influence on the colloidal stability of NP. At pH values of the test media (adjusted with NaOH and HCl without additional salts) no aggregation of DMSA-IONP was observed (ESI† Table S3). On the other hand uncoated IONP showed strong aggregation at all three pH values (ESI† Table S3). Therefore, the decreased colloidal stability of DMSA-IONP in LM and DM might be mainly due to the presence of high amounts of divalent cations in the media, while for uncoated IONP, their aggregation appears to be the combined effects of pH and divalent cations.

3.3 Toxic effects of IONP on algae, *Lemna* and *Daphnia*

Effects of DMSA-IONP, fresh and aged uncoated IONP in the concentration range of $0.18\text{--}1791 \text{ }\mu\text{M Fe}$ ($0.01\text{--}100 \text{ mg Fe L}^{-1}$) on algae, *Lemna* and *Daphnia* were investigated, and the EC_{50} values were determined (Table 4). Algal growth was totally inhibited by the three types of IONP when the concentration was $\geq 17.91 \text{ }\mu\text{M Fe}$ (1.0 mg Fe L^{-1} , Fig. 4A). The EC_{50} of DMSA-IONP for the algal growth after 72 h was $2.27 \pm 0.19 \text{ }\mu\text{M Fe}$ ($0.13 \pm 0.01 \text{ mg Fe L}^{-1}$), while fresh and aged uncoated IONP showed stronger effects on algae, with EC_{50} values of 1.62 and $0.86 \text{ }\mu\text{M Fe}$ (0.09 and $0.05 \text{ mg Fe L}^{-1}$), respectively. None of the IONP had significant effects on the growth rate of *Lemna* within the tested concentration range and the test duration of 7 days (Fig. 4B). For *Daphnia*, no significant immobilization was observed when exposed to DMSA-IONP for 72 h ($\text{NOEC} > 1791 \text{ }\mu\text{M Fe}$, *i.e.* 100 mg Fe L^{-1}), but significant toxicity was found when the *Daphnia* were exposed to fresh ($p < 0.01$) or aged ($p < 0.001$) uncoated IONP at concentrations $\geq 179.1 \text{ }\mu\text{M Fe}$ (10 mg Fe L^{-1} , Fig. 4C) with up to 30–50% inhibition.

Pure DMSA was also tested (Fig. 4D) to consider potential ecotoxicological effects that might be caused by unbound or

Table 3 Main components, total ionic strength (IS) and concentration of divalent cations (Ca^{2+} , Mg^{2+}) in the three media for testing. IS was calculated with software PHREEQCi (v.3)⁴¹

Medium	Main components	Total ionic strength [mM]	Divalent cations (Ca^{2+} , Mg^{2+}) [mM]
OECD TG 201 (<i>R. subcapitata</i>) pH 8.1	NaHCO_3 , NH_4Cl , CaCl_2 , MgSO_4	1.7	0.24
ISO 6341 (<i>D. magna</i>) pH 7.0	CaCl_2 , MgSO_4 , NaHCO_3 , KCl	8.4	2.5
OECD 221 Steinberg (<i>L. minor</i>) pH 5.5	KNO_3 , $\text{Ca}(\text{NO}_3)_2$, MgSO_4 , KH_2PO_4	9.3	1.66

Table 4 EC_{50} values of DMSA-IONP, uncoated IONP and pure DMSA for algae, *Lemna* and *Daphnia*. (-) indicates data not available

Substance	Algae		<i>Lemna</i>		<i>Daphnia</i>	
	EC_{50} values in $\mu\text{M Fe}$ and mg Fe L^{-1} (confidence interval)					
	μM	mg L^{-1}	μM	mg L^{-1}	μM	mg L^{-1}
Fresh uncoated IONP	1.62 (-)	0.09 (-)	>1791	>100	1181 (298–18 771)	65.94 (16.67–1048)
Aged uncoated IONP	0.86 (-)	0.05 (-)	>1791	>100	374 (100–2870)	20.89 (5.60–160.28)
DMSA-IONP	2.27 (2.10–2.47)	0.13 (0.12–0.14)	>1791	>100	>1791	>100
Pure DMSA	31.42 (16.56–60.03)	5.73 (3.02–10.94)	>179.1	>32.6	>179.1	>32.6



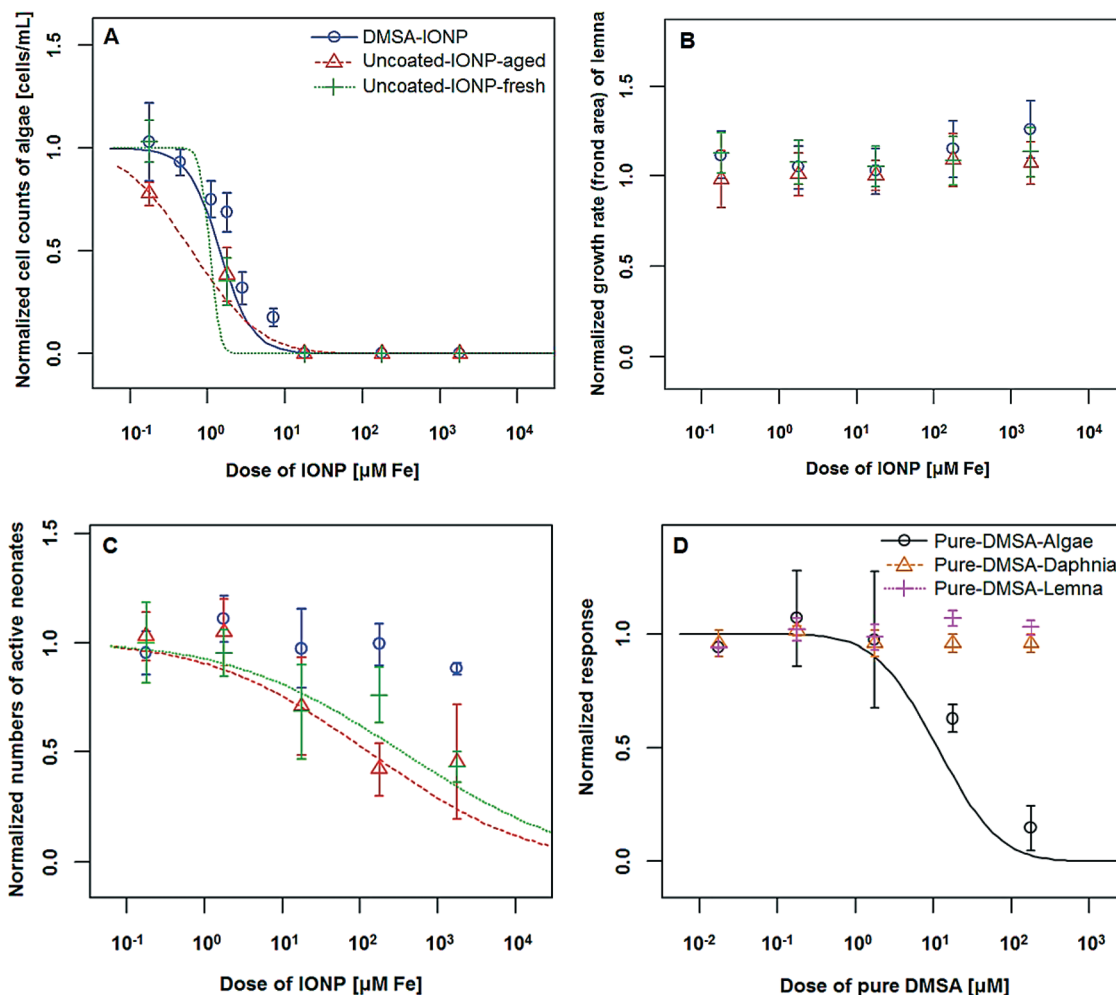


Fig. 4 Dose–response curves of DMSA-IONP, fresh and aged uncoated IONP for *R. subcapitata* (A), *L. minor* (B) and *D. magna* (C). Dose–response curves of pure DMSA for all three test organisms (D). Plots were established by linlogit or probit model of ‘drfit’ package in R. Data represent mean values \pm SD ($n = 12, 16, 40$ or 6).

released coating material. No adverse effect of pure DMSA towards *Lemna* and *Daphnia* in the tested concentration range was observed, but a significant inhibition on algal growth was determined for DMSA at concentrations $>1.79 \mu\text{M}$ (0.326 mg L^{-1}). The effect at high concentration might be attributed to the chelating effect of DMSA molecules^{15,16,18} and the masking (removal) of trace elements such as Fe^{3+} or Zn^{2+} from the algae medium. The concentration of DMSA in the IONP dispersion is unknown, but based on the synthesis, a concentration between $3.26 \mu\text{g L}^{-1}$ to 32.6 mg L^{-1} DMSA is assumed (this corresponds to one-tenth with regard to the iron content of the IONP, *i.e.* $0.01\text{--}100 \text{ mg Fe L}^{-1}$). Due to the high colloidal stability of DMSA-IONP dispersions (Table 2 and ESI† Fig. S2) in algae medium, an almost complete release of DMSA in the algae medium appears unlikely. Therefore, the contribution of free/released DMSA to the toxicity of DMSA-IONP on algae is assumed to be negligible.

3.3.1 Effect of IONP on *R. subcapitata*. DMSA- and uncoated IONP exhibited strong inhibition to algal growth. Intact algae cells (Fig. 5A) were hardly observed after 72 h exposure to DMSA- or uncoated IONP at a concentration of

$1791 \mu\text{M Fe}$ (100 mg Fe L^{-1}) (Fig. 5B and C). DMSA-IONP exhibited a high colloidal stability in algae medium (Table 2 and ESI† Fig. S2) while uncoated IONP precipitated quickly, but still exhibited similar toxicity. In both cases a flocculation of algae cells was observed (Fig. 5B and C) which increased the cellular weight and supported the cell (co-)sedimentation. Moreover, the flocculation is known not only to hamper access to nutrients and light,^{48,49} but also to interrupt the gas exchange.⁴⁸ A previous study has reported similar results when *P. subcapitata* were exposed to TiO_2 NP:⁵⁰ large

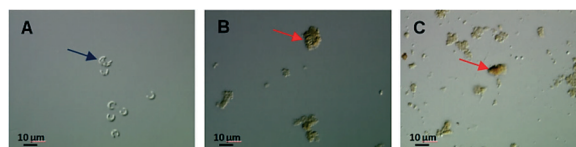


Fig. 5 Microscopic pictures of *R. subcapitata* (magnification: 400). Controls (A), exposure to DMSA-IONP (B) and uncoated IONP (C) in concentration of $1791 \mu\text{M Fe}$ (100 mg Fe L^{-1}) after 72 h. Blue and red arrows indicate untreated algae and algae-IONP flocculation respectively.



aggregates entrapped almost all algae cells and high toxicity was observed after 72 h.

The influence of iron ions on algal growth was further investigated (Fig. 6), aiming to compare and discriminate between the effects of iron ions and particulate IONP. Potentially released iron can occur as ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. Fe^{2+} is assumed to be fully oxidized to Fe^{3+} in the presence of O_2 (available in the media). The assumption is supported by speciation calculations (ESI† Table S4). Since these ions might contribute to the observed toxic effects we tested ferric ammonium citrate (Fe^{3+} , FAC) and ferrous ammonium sulfate (Fe^{2+} , FAS) in the same concentration range as IONP (0.18–1791 μM Fe, *i.e.* 0.01–100 mg Fe L^{-1}) for potential adverse effects on algae (Fig. 6). In a test performed with standard medium that contains 0.24 μM Fe^{3+} (and EDTA) the two salts stimulated algal growth significantly at concentrations ≤ 82.4 and 3.9 μM Fe (4.6 and 0.22 mg Fe L^{-1}), respectively (Fig. 6A). Only at concentrations ≥ 386.8 and 17.9 μM

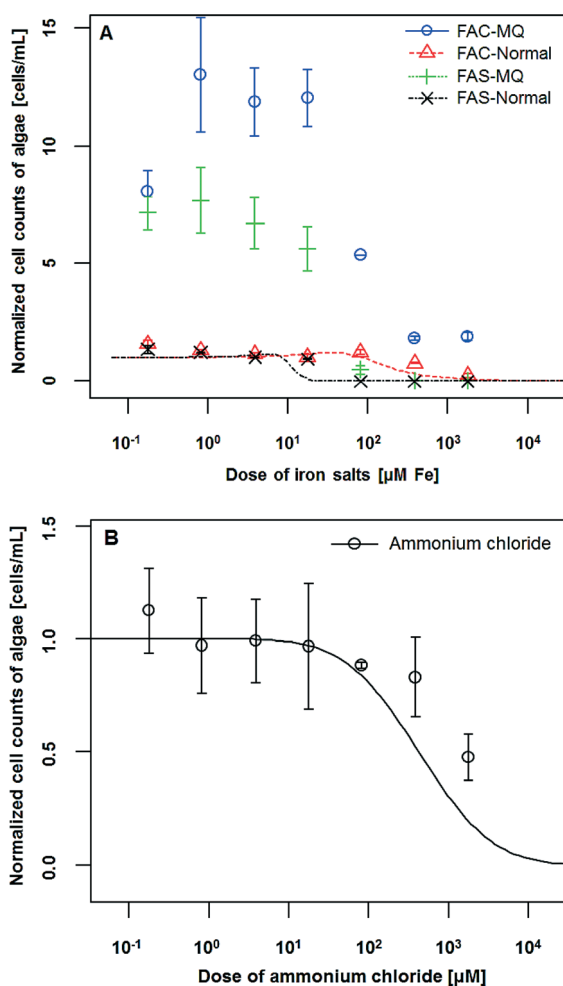


Fig. 6 Effects of (A) ferric ammonium citrate (FAC, Fe^{3+}), ferrous ammonium sulfate (FAS, Fe^{2+}) and (B) ammonium chloride on the growth of *R. subcapitata* after 72 h. 'Normal' and 'MQ' indicate the standard and iron-free algae medium, respectively. The plots were established by probit or linlogit model of 'drfit' package in R. Data shown represent mean values \pm SD ($n = 9$ or 3).

Fe (21.6 and 1.0 mg Fe L^{-1}) could toxic effects of Fe^{3+} and Fe^{2+} be observed with a calculated EC_{50} of 689.2 and 24.5 μM Fe (38.5 and 1.4 mg Fe L^{-1}), respectively. Similar experiments were conducted, but with an iron-free medium. Here the addition of ferric and ferrous species caused 5–12 times higher cell counts (Fig. 6A). Under this condition, even a high concentration of Fe^{3+} (1791 μM Fe, *i.e.* 100 mg Fe L^{-1}) greatly promoted algal growth ($\text{EC}_{50} > 1791 \mu\text{M Fe}$, *i.e.* 100 mg Fe L^{-1}), while the Fe^{2+} only supported the growth at concentrations $\leq 17.91 \mu\text{M}$ (1.0 mg Fe L^{-1}) with an EC_{50} of 82.4 μM Fe (4.6 mg Fe L^{-1}). When algae were exposed to higher concentrations of Fe^{2+} , growth was totally inhibited in both types (standard and iron-free) of algae media. The accelerated growth stimulation by FAC may be due to the combined effects of the presence of both Fe^{3+} and citrate. Citrates as complex agents have been reported to enhance availability of metal nutrients to algae⁵¹ and stimulate growth.⁵² Moreover, growth stimulation in the presence of iron ions is expected, since iron is the most important trace component for optimum growth of algae⁵³ by involving in photosynthetic carbon and nitrogen reduction.⁵⁴

In addition, the influence of another component (ammonium tested as NH_4Cl) in FAC and FAS on algal growth was also investigated (Fig. 6B) in a concentration range of 0.18–1791 μM considering its important role as a source of nitrogen in plant growth.⁵⁴ However, no significant stimulation was observed at concentrations of 386.9 μM or lower. Thus the contribution of ammonium ions in promoting algal growth in the presence of FAC/FAS can be neglected.

In subsequent experiments the toxicity of DMSA-coated and uncoated IONP in an iron-free medium was investigated (Fig. 7). Here the only source of iron is IONP which are presented in low concentrations of 1.8×10^{-5} –17.91 μM Fe (equal to 1.0×10^{-6} –1.0 mg Fe L^{-1}). An additional potential iron ion source, such as DMSA-coated or uncoated IONP, was

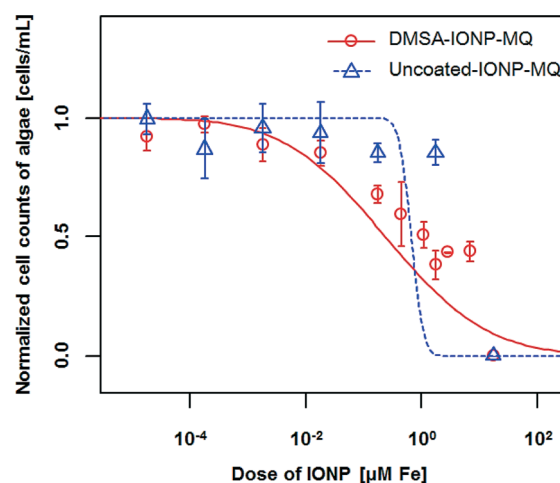


Fig. 7 *R. subcapitata* exposed to DMSA-coated or fresh uncoated IONP, containing the extremely low concentrations (as low as 18 pM, *i.e.* 1.0×10^{-6} mg Fe L^{-1}), in the iron-free medium after 72 h. 'MQ' indicates the iron-free algae medium. Data shown represent mean values \pm SD ($n = 6$).



expected to promote algal growth at low concentrations if iron ions are released. However, neither DMSA-coated nor uncoated IONP significantly increased the algal growth through the tested concentrations. DMSA-IONP of concentrations $\leq 1.79 \mu\text{M Fe}$ (0.1 mg Fe L^{-1}) caused a greater suppression on algal growth in the iron-free medium (Fig. 7, red curve) when compared with standard medium (Fig. 4A). Generally, a significantly ($p < 0.01$) decreased EC_{50} value of DMSA-IONP in the iron-free medium was observed ($0.85 \pm 0.40 \mu\text{M Fe}$, *i.e.* $0.05 \pm 0.02 \text{ mg Fe L}^{-1}$). Nevertheless, in this case, slightly but significantly decreased toxicity was found at 7.13 and $2.85 \mu\text{M Fe}$ (0.4 and $0.16 \text{ mg Fe L}^{-1}$) compared to the standard medium. In comparison to DMSA-IONP, uncoated IONP (Fig. 7, blue curve) in the iron-free medium at concentrations of 1.79 and $0.18 \mu\text{M Fe}$ (0.1 and $0.01 \text{ mg Fe L}^{-1}$) showed lower toxic effects on the algal growth and the cell counts almost reached the control value.

The results indicate that free Fe-species are released from DMSA-coated and uncoated IONP, with higher amounts in the latter due to their comparatively lower stability in algae medium. It might also be possible that the observed stronger inhibition induced by DMSA-IONP is due to the decreased availability (chelation/adsorption) of potentially released iron ions or other trace elements from the medium by DMSA molecules. However, apparently the positive effects of liberated iron ions are compensated by adverse effects of particulate IONP *per se*, thus strong enhancement in growth is unlikely to be observed.

3.3.2 Effect of IONP on *L. minor*. All three types of IONP were non-toxic to *Lemna*, probably due to the strong aggregation of IONP in the testing solutions (Table 2 and Fig. 3), which leads to low availability of IONP to *Lemna* cells. Particle size and colloidal stability are important factors in determining bioavailability in the aqueous scenario. Dispersed NP have more chances to get in contact with cells,⁵⁵ whereas aggregated NP have smaller surface-to-volume ratios, leading to lower capacity to be absorbed or to pass through the surface of organisms.⁵⁶ During the exposure of *Lemna* to IONP, some aggregated IONP were found adsorbed on the root surface of *Lemna* colonies (Fig. 8B and C). Recently, a high degree of aggregation was also observed for CuO NP: the lower uptake of these NP compared to poly(styrene-*co*-butyl acrylate) (CS) coated-CuO NP, which had higher colloidal stability in the medium, was suggested as main reason for the low toxicity of

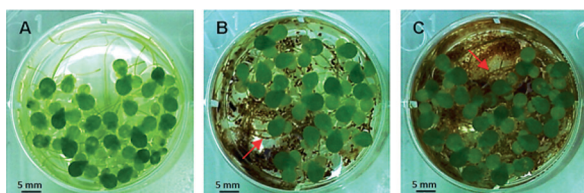


Fig. 8 Visual observation of *L. minor*. (A) In OECD TG 221 medium, and exposed to (B) DMSA-IONP, (C) uncoated IONP in a concentration of $1791 \mu\text{M Fe}$ (100 mg Fe L^{-1}) after 7 days. Red arrow indicates adsorbed DMSA-coated or uncoated IONP.

CuO NP to *L. gibba*.⁵⁷ Moreover, *Lemna* could absorb large amounts of nutrients directly through the lower surface of the floating fronds when the nutrient uptake through roots was limited, *e.g.* when NP were adsorbed on the surface.⁵⁸

In terms of bioavailability it was also expected that the algae would be more susceptible to IONP than *Lemna*. The surface area to volume ratio of the cell itself is evaluated as 0.9^{59} (surface area⁶⁰ = $66.96 \mu\text{m}^2$, biovolume⁶⁰ = $74.49 \mu\text{m}^3$) for *R. subcapitata*, several orders of magnitude higher than for *Lemna* (4×10^{-6}).⁵⁹

3.3.3 Effect of IONP on *D. magna*. After a 72 h exposure, ingestion and accumulation of IONP in the gastrointestinal tract (GIT) of *Daphnia* was visually observed (Fig. 9). Similar accumulation in GIT and some toxicity of NP to *Daphnia* has also been reported for TiO_2 ,^{61,62} C_{60} ,⁶¹ iron³² and gold⁶³ NP. *Daphnia* are filter feeders, and they collect food by a filtering apparatus called phylo-pods (flattened leaf-like legs) to produce water current. *D. magna* have been reported to be able to filter particles in a size range of 0.2^{64} – $50^{65} \mu\text{m}$. The IONP aggregates formed in the present study are within the size range of the food particles. Accumulation of DMSA-IONP in GIT (Fig. 9B), however, did not induce significant toxicity of DMSA-IONP (as high as $1791 \mu\text{M Fe}$, *i.e.* 100 mg Fe L^{-1}) after 72 h. One reason could be the DMSA coating prevented direct contact of the IONP cores and in this way reducing ROS production and physical damage to *Daphnia* tissue. PVP-IONP (Fe_3O_4) were also reported to have low toxicity to *Daphnia* with EC_{50} higher than 100 mg Fe L^{-1} , which was attributed to their high colloidal stability without releasing iron ions,³² and to avoiding the neonates being affected by agglomerates or IONP adsorbed to the carapace or the gills in large quantities.³² *Daphnia* exposed to uncoated IONP accumulated NP not only in GIT, but also in the filtering apparatus, as well as at the exoskeleton and the surface of the antenna and apical spine (Fig. 9C). Such adhesion has also been reported when *Daphnia* were exposed to TiO_2 and C_{60} NP.⁶¹ The higher body-burden of *Daphnia* due to uncoated IONP intake and adhesion further increases the weight and physical restraint during swimming, so excess energy is needed not only for movements, but also for increasing

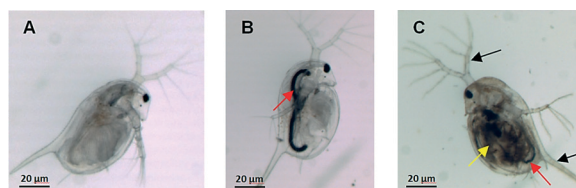


Fig. 9 Visual observation of *D. magna* by stereo zoom microscope (A) in the ISO 6341 medium, exposed to (B) DMSA-IONP or (C) uncoated IONP in a concentration of $1791 \mu\text{M Fe}$ (100 mg Fe L^{-1}) after 72 h. Arrows indicate IONP. (B) High level of DMSA-IONP accumulation in the digestive tract of a neonate (red arrow). (C) Uncoated IONP are not only strongly accumulated in the digestive tract (red arrow), but also accumulated in the filter apparatus (yellow arrow). Adsorbed uncoated IONP are also visible on the surface of the antenna and apical spine (black arrows).



filtration rates⁶⁶ and evacuating these particles.⁶³ This loss in energy would also negatively influence the normal growth of *Daphnia*, which could be one reason for the significant toxicity of uncoated IONP at concentrations $\geq 179.1 \mu\text{M Fe}$ (10 mg Fe L^{-1}). Moreover, potentially released iron ions (as discussed for algae) might contribute to the observed toxic effects. For instance Fe^{3+} is known to cause toxic effects towards *Daphnia magna* (48 h immobilization test) at quite low concentrations ($\text{EC}_{50} = 2.3 \text{ mg Fe L}^{-1}$).⁶⁷ Besides, molting disruption induced by e.g. TiO_2 NP is considered as an important factor for the high levels of immobility and mortality of *D. magna*.⁶⁸ Inhibition of molting and corresponding toxicity was also observed when exposing *Daphnia* to citrate-IONP after 72 h,³² but no such observation was found during the 72 h incubation (Fig. 9B and C) in the present study.

4. Conclusions

In order to assess the aquatic toxicity of IONP we have shown that DMSA-IONP, as well as fresh and aged uncoated IONP, cause strong, short-term toxicological effects to green algae *R. subcapitata*. The EC_{50} values for the three types of IONP ranged between $0.86\text{--}2.27 \mu\text{M Fe}$ (0.05 to $0.13 \text{ mg Fe L}^{-1}$). So far no negative effects of IONP have been reported to algae. A recent study investigating two types of zero-valent IONP, bare and coated with Na-acrylic copolymer, has proved that NP support the growth of marine microalgae *P. lutheri* and *I. galbana*.⁶⁹ It has been assumed that this effect is due to an enhanced bioavailability of iron in the form of NP. Our study with DMSA-IONP suggests that neither potentially released $\text{Fe}^{2+}/\text{Fe}^{3+}$ nor DMSA molecules from the coating are responsible for the observed toxic effects. Toxicity seems to be caused by interactions of algae cells with particulate IONP. The low colloidal stability of the uncoated IONP in algae medium comes with a rapid precipitation of particles and leads to a co-sedimentation of algae cells, whereas DMSA-IONP exhibit a much higher colloidal stability, but cause the flocculation of algae cells. In both cases the reduced access to nutrients and light might be mainly responsible for the growth inhibition. Moreover, the evolution of ROS (likely to be evolved if iron ions are released) and the NP-corona formed by biomolecules secreted by organisms might be important to explain observed toxic effects, but have not been investigated within this study.

None of the investigated particles showed effects on *L. minor* ($\text{EC}_{50\text{s}} > 1791 \mu\text{M Fe}$, i.e. 100 mg Fe L^{-1}), and only for uncoated IONP could minor effects to *D. magna* be observed ($\text{EC}_{50\text{s}} > 179.1 \mu\text{M Fe}$, i.e. 10 mg Fe L^{-1}). However, the ingestion and accumulation of DMSA-coated and uncoated IONP into the gastrointestinal tract was observed. Uncoated IONP were even found at the filtering apparatus, the surface of antenna and the apical spine, which is assumed to hamper the normal growth of *Daphnia* by decreasing their mobility.

The visible high body-burden in *Daphnia* might not highly affect their mobility in short-term testing, but most likely will have an influence on chronic toxicity, as it was recently dem-

onstrated that the immobilizing effect of ascorbate-, citrate- and dextran-IONP (Fe_3O_4) on *D. magna* increased during a slightly prolonged exposure for 96 h.³² Moreover, considering the accumulation of IONP in *Daphnia*, organisms at higher trophic levels may be negatively affected due to bio-magnification. Given the possibility of IONP affecting aquatic organisms at both individual and population levels, due to not only short-term but also potential long-term exposure, there might be risks to the aquatic ecosystem through application of these NP in water treatment and environmental remediation, which perhaps should be kept in mind. Since there have been no major releases of IONP, due to its not having been widely applied in the real world, little knowledge is available on the distribution level of IONP in aquatic environment.³¹ Related but too limited information so far is available for zero-valent iron nanoparticles (nZVI), which have been used to remediate contaminated soil and groundwater for decades. Grieger *et al.* summarized the applied concentrations of nZVI used for environmental remediation at some documented sites in the USA.⁷⁰ The concentrations ranged from 0.75 to 140 g L^{-1} which were much higher than the $\text{EC}_{50\text{s}}$ of the IONP to *Raphidocelis* and *Daphnia* in the present study. Although the high concentrations applied were on land, subsequent release of these NP to the aquatic environment is highly likely. The present study covered a wide concentration range of IONP, mainly from 1.0×10^{-6} to 100 mg Fe L^{-1} , which was supposed to provide some information on the acceptable exposure levels of some basic aquatic organisms (including the primary producers) to these IONP. In addition, data presented here were based on laboratory standard tests which were performed in 'ideal' conditions, and IONP would experience physical and chemical transformations in the real environment which may further influence their effects on aquatic organisms. The strong negative effects on algae *Raphidocelis* observed here do raise concerns as these will have pronounced cascade effects on higher trophic levels, due to food limitation. Therefore, in remediation scenarios, benefits of IONP by detoxification should be viewed in comparison to negative side effects *via* the aquatic food chain.

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Notes and references

- 1 D. L. Huber, Synthesis, properties, and applications of iron nanoparticles, *Small*, 2005, 1, 482–501.
- 2 A. K. Gupta and M. Gupta, Synthesis and surface engineering



- of iron oxide nanoparticles for biomedical applications, *Biomaterials*, 2005, 26, 3995–4021.
- 3 A. B. Cundy, L. Hopkinson and R. L. D. Whitby, Use of iron-based technologies in contaminated land and groundwater remediation: a review, *Sci. Total Environ.*, 2008, 400, 42–51.
 - 4 P. Xu, G. M. Zeng, D. L. Huang, C. L. Feng, S. Hu, M. H. Zhao, C. Lai, Z. Wei, C. Huang, G. X. Xie and Z. F. Liu, Use of iron oxide nanomaterials in wastewater treatment: a review, *Sci. Total Environ.*, 2012, 424, 1–10.
 - 5 N. N. Nassar, Rapid removal and recovery of Pb(II) from wastewater by magnetic nano-adsorbents, *J. Hazard. Mater.*, 2010, 184, 538–546.
 - 6 J. Hu, G. Chen and I. M. C. Lo, Removal and recovery of Cr(VI) from wastewater by maghemite nanoparticles, *Water Res.*, 2005, 39, 4528–4536.
 - 7 L. Carlos, F. S. G. Einschlag, M. C. González and D. O. Mártire, Applications of magnetite nanoparticles for heavy metal removal from wastewater, *Waste Water Treat. Technol. Recent Anal. Dev.*, INTECH Open Access Publisher, 2013, pp. 63–78.
 - 8 D. Maity and D. C. Agrawal, Synthesis of iron oxide nanoparticles under oxidizing environment and their stabilization in aqueous and non-aqueous media, *J. Magn. Mater.*, 2007, 308, 46–55.
 - 9 J. Filser, D. Arndt, J. Baumann, M. Geppert, S. Hackmann, E. M. Luther, C. Pade, K. Prenzel, H. Wigger, J. Arning, M. C. Hohnholt, K. Köser, A. Kück, E. Lesnikov, J. Neumann, S. Schüttrumpf, J. Warrelmann, M. Bäumer, R. Dringen, A. von Gleich, P. Swiderek and J. Thöming, Intrinsically green iron oxide nanoparticles? From synthesis via (eco-)toxicology to scenario modelling, *Nanoscale*, 2013, 5, 1034–1046.
 - 10 M. Chen, S. Yamamuro, D. Farrell and S. A. Majetich, Gold-coated iron nanoparticles for biomedical applications, *J. Appl. Phys.*, 2003, 93, 7551.
 - 11 C. Tassa, S. Y. Shaw and R. Weissleder, Dextran-coated iron oxide nanoparticles: a versatile platform for targeted molecular imaging, molecular diagnostics, and therapy, *Acc. Chem. Res.*, 2011, 44, 842–852.
 - 12 J. Liu, Z. Zhao and G. Jiang, Coating Fe₃O₄ magnetic nanoparticles with humic acid for high efficient removal of heavy metals in water, *Environ. Sci. Technol.*, 2008, 42, 6949–6954.
 - 13 C. R. A. Valois, J. M. Braz, E. S. Nunes, M. A. R. Vinolo, E. C. D. Lima, R. Curi, W. M. Kuebler and R. B. Azevedo, The effect of DMSA-functionalized magnetic nanoparticles on transendothelial migration of monocytes in the murine lung via a β_2 integrin-dependent pathway, *Biomaterials*, 2010, 31, 366–374.
 - 14 W. Yantasee, C. L. Warner, T. Sangvanich, R. S. Addleman, T. G. Carter, R. J. Wiacek, G. E. Fryxell, C. Timchalk and M. G. Warner, Removal of heavy metals from aqueous systems with thiol functionalized superparamagnetic nanoparticles, *Environ. Sci. Technol.*, 2007, 41, 5114–5119.
 - 15 Thorne Research, Inc., Dimercaptosuccinic Acid, *Altern. Med. Rev. Monogr.*, 2002, pp. 132–136.
 - 16 K. Sompamit, U. Kukongviriyapan, W. Donpunha, S. Nakmareong and V. Kukongviriyapan, Reversal of cadmium-induced vascular dysfunction and oxidative stress by meso-2,3-dimercaptosuccinic acid in mice, *Toxicol. Lett.*, 2010, 198, 77–82.
 - 17 C. Petters, E. Irrsack, M. Koch and R. Dringen, Uptake and metabolism of iron oxide nanoparticles in brain cells, *Neurochem. Res.*, 2014, 39, 1648–1660.
 - 18 H. V. Aposhian, DMSA and DMPS—water soluble antidotes for heavy metal poisoning, *Annu. Rev. Pharmacol. Toxicol.*, 1983, 23, 193–215.
 - 19 M. E. Sears, Chelation: Harnessing and enhancing heavy metal detoxification - A review, *Sci. World J.*, 2013, 2013, 219840, DOI: 10.1155/2013/219840.
 - 20 D. Mishra and S. J. S. Flora, Differential oxidative stress and DNA damage in rat brain regions and blood following chronic arsenic exposure, *Toxicol. Ind. Health*, 2008, 24, 247–256.
 - 21 N. Ercal, P. Treeratphan, T. C. Hammond, R. H. Matthews, N. H. Grannemann and D. R. Spitz, In vivo indices of oxidative stress in lead-exposed C57BL/6 mice are reduced by treatment with meso-2,3-dimercaptosuccinic Acid or N-acetylcysteine, *Free Radical Biol. Med.*, 1996, 21, 157–161.
 - 22 N. Fauconnier, J. N. Pons, J. Roger and A. Bee, Thiolation of maghemite nanoparticles by dimercaptosuccinic acid, *J. Colloid Interface Sci.*, 1997, 194, 427–433.
 - 23 H. Muckter, B. Lieb, F.-X. Reich, G. Hunder, U. Walther and B. Fichtl, Are we ready to replace dimercaprol (BAL) as an arsenic antidote, *Hum. Exp. Toxicol.*, 1997, 16, 460–465.
 - 24 H. V. Aposhian, D. E. Carter, T. D. Hoover, C. A. Hsu, R. M. Maiorino and E. Stine, DMSA, DMPS, and DMPA-as arsenic antidotes, *Toxicol. Sci.*, 1984, 4, 58–70.
 - 25 H. V. Aposhian, R. M. Maiorino, D. Gonzalez-ramirez, M. Zuniga-charles, Z. Xu, K. M. Hurlbut, P. Junco-munoz, R. C. Dart and M. M. Aposhian, Mobilization of heavy metals by newer, therapeutically useful chelating agents, *Toxicology*, 1995, 97, 23–38.
 - 26 S. Singh, K. C. Barick and D. Bahadur, Surface engineered magnetic nanoparticles for removal of toxic metal ions and bacterial pathogens, *J. Hazard. Mater.*, 2011, 192, 1539–1547.
 - 27 C. L. Warner, R. S. Addleman, A. D. Cinson, T. C. Droubay, M. H. Engelhard, M. A. Nash, W. Yantasee and M. G. Warner, High-performance, superparamagnetic, nanoparticle-based heavy metal sorbents for removal of contaminants from natural waters, *ChemSusChem*, 2010, 3, 749–757.
 - 28 M. Geppert, M. C. Hohnholt, K. Thiel, S. Nürnberger, I. Grunwald, K. Rezwan and R. Dringen, Uptake of dimercaptosuccinate-coated magnetic iron oxide nanoparticles by cultured brain astrocytes, *Nanotechnology*, 2011, 22, 145101.
 - 29 M. Auffan, L. Decome, J. Rose, T. Orsiere, M. De Meo, V. Briois, C. Chaneac, L. Olivi, J. Berge-lefranc, A. Botta, M. R. Wiesner and J. Bottero, In vitro interactions between DMSA-coated maghemite nanoparticles and human fibroblasts: a physicochemical and cyto-genotoxic study, *Environ. Sci. Technol.*, 2006, 40, 4367–4373.



- 30 B. Wang, W. Feng, M. Zhu, Y. Wang, M. Wang, Y. Gu, H. Ouyang, H. Wang, M. Li, Y. Zhao, Z. Chai and H. Wang, Neurotoxicity of low-dose repeatedly intranasal instillation of nano- and submicron-sized ferric oxide particles in mice, *J. Nanopart. Res.*, 2009, **11**, 41–53.
- 31 X. Zhu, S. Tian and Z. Cai, Toxicity assessment of iron oxide nanoparticles in zebrafish (*Danio rerio*) early life stages, *PLoS One*, 2012, **7**, 1–6.
- 32 J. Baumann, J. Köser, D. Arndt and J. Filser, The coating makes the difference: acute effects of iron oxide nanoparticles on *Daphnia magna*, *Sci. Total Environ.*, 2014, **484**, 176–184.
- 33 OECD, Test No. 201: freshwater alga and cyanobacteria, growth inhibition test, *OECD guidelines for the testing of chemicals*, 2011, section 2, DOI: 10.1787/9789264069923-en.
- 34 OECD, Test No. 221: lemna sp. growth inhibition test, *OECD guidelines for the testing of chemicals*, 2006, section 2, DOI: 10.1787/9789264016194-en.
- 35 MicroBioTests Inc., Daphtokit F™ magna, <http://www.microbiotests.be/toxkits/daphtokitf.pdf>.
- 36 A. Bee, R. Massart and S. Neveu, Synthesis of very fine maghemite particles, *J. Magn. Magn. Mater.*, 1995, **149**, 6–9.
- 37 M. Geppert, M. Hohnholt, L. Gaetjen, I. Grunwald, M. Bäumer and R. Dringen, Accumulation of iron oxide nanoparticles by cultured brain astrocytes, *J. Biomed. Nanotechnol.*, 2009, **5**, 285–293.
- 38 J. Riemer, H. H. Hoepken, H. Czerwinska, S. R. Robinson and R. Dringen, Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells, *Anal. Biochem.*, 2004, **331**, 370–375.
- 39 W. Drost, M. Matzke and T. Backhaus, Heavy metal toxicity to *Lemna minor*: studies on the time dependence of growth inhibition and the recovery after exposure, *Chemosphere*, 2007, **67**, 36–43.
- 40 J. Baumann, Y. Sakka, C. Bertrand, J. Köser and J. Filser, Adaptation of the *Daphnia sp.* acute toxicity test: miniaturization and prolongation for the testing of nanomaterials, *Environ. Sci. Pollut. Res.*, 2014, **21**, 2201–2213.
- 41 S. R. Charlton and D. L. Parkhurst, *Phreeqci - A Graphical User Interface for the Geochemical Computer Program PHREEQC*, U.S. Geological Survey, 2016, Available from: http://wwwbr.cr.usgs.gov/projects/GWC_coupled/phreeqci/index.html.
- 42 H. D. Schulz and M. Kölling, *DVWK-Schriften 100: Anwendung hydrogeochemischer Modelle*, Verlag Paul Parey, Hamburg and Berlin, 1992, p. 45.
- 43 M. Pavlin and V. B. Bregar, Stability of nanoparticle dispersions in different biologically relevant media, *Dig. J. Nanomater. Biostruct.*, 2012, **7**, 1389–1400.
- 44 Z. Ji, X. Jin, S. George, T. Xia, H. Meng, X. Wang, E. Suarez, H. Zhang, E. M. V. Hoek, H. Godwin, A. E. Nel and J. I. Zink, Dispersion and stability optimization of TiO₂ nanoparticles in cell culture media, *Environ. Sci. Technol.*, 2010, **44**, 7309–7314.
- 45 K. A. Huynh and K. L. Chen, Aggregation kinetics of citrate and polyvinylpyrrolidone coated silver nanoparticles in monovalent and divalent electrolyte solutions, *Environ. Sci. Technol.*, 2011, **45**, 5564–5571.
- 46 S. L. Chinnapongse, R. I. MacCuspie and V. A. Hackley, Persistence of singly dispersed silver nanoparticles in natural freshwaters, synthetic seawater, and simulated estuarine waters, *Sci. Total Environ.*, 2011, **409**, 2443–2450.
- 47 C. O. Metin, L. W. Lake, C. R. Miranda and Q. P. Nguyen, Stability of aqueous silica nanoparticle dispersions, *J. Nanopart. Res.*, 2011, **13**, 839–850.
- 48 E. Navarro, A. Baun, R. Behra, N. B. Hartmann, J. Filser, A.-J. Miao, A. Quigg, P. H. Santschi and L. Sigg, Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi, *Ecotoxicology*, 2008, **17**, 372–386.
- 49 C. Wei, Y. Zhang, J. Guo, B. Han, X. Yang and J. Yuan, Effects of silica nanoparticles on growth and photosynthetic pigment contents of *Scenedesmus obliquus*, *J. Environ. Sci.*, 2010, **22**, 155–160.
- 50 V. Aruoja, H.-C. Dubourguier, K. Kasemets and A. Kahru, Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*, *Sci. Total Environ.*, 2009, **407**, 1461–1468.
- 51 O. Errecalde, M. Seidl and P. G. C. Campbell, Influence of a low molecular weight metabolite (citrate) on the toxicity of cadmium and zinc to the unicellular green alga *Selenastrum capricornutum*: an exception to the free-ion model, *Water Res.*, 1998, **32**, 419–429.
- 52 W. A. Kratz and J. Myers, Nutrition and growth of several blue-green algae, *Am. J. Bot.*, 1955, **42**, 282–287.
- 53 M. Chen, H. Tang, H. Ma, T. C. Holland, S. K. Y. Ng and S. O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, *Bioresour. Technol.*, 2011, **102**, 1649–1655.
- 54 C. Reynolds, *Ecology of Phytoplankton*, Cambridge University Press, 2006.
- 55 Z. Wang, J. Li, J. Zhao and B. Xing, Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter, *Environ. Sci. Technol.*, 2011, **45**, 6032–6040.
- 56 G. Song, Y. Gao, H. Wu, W. Hou, C. Zhang and H. Ma, Physiological effect of anatase TiO₂ nanoparticles on *Lemna minor*, *Environ. Toxicol. Chem.*, 2012, **31**, 2147–2152.
- 57 F. Perreault, R. Popovic and D. Dewez, Different toxicity mechanisms between bare and polymer-coated copper oxide nanoparticles in *Lemna gibba*, *Environ. Pollut.*, 2014, **185**, 219–227.
- 58 G. Juhel, E. Batisse, Q. Hugues, D. Daly, F. N. A. M. van Pelt, J. O'Halloran and M. A. K. Jansen, Alumina nanoparticles enhance growth of *Lemna minor*, *Aquat. Toxicol.*, 2011, **105**, 328–336.
- 59 K. K. Jessing, M. Andresen and N. Cedergreen, Temperature-dependent toxicity of artemisinin toward the macrophyte *Lemna minor* and the algae *Pseudokirchneriella subcapitata*, *Water, Air, Soil Pollut.*, 2014, **225**, 2010.
- 60 J. A. Weiner, M. E. DeLorenzo and M. H. Fulton, Relationship between uptake capacity and differential toxicity of the herbicide atrazine in selected microalgal species, *Aquat. Toxicol.*, 2004, **68**, 121–128.



- 61 A. Baun, N. B. Hartmann, K. Grieger and K. O. Kusk, Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing, *Ecotoxicology*, 2008, 17, 387–395.
- 62 X. Zhu, Y. Chang and Y. Chen, Toxicity and bioaccumulation of TiO₂ nanoparticle aggregates in *Daphnia magna*, *Chemosphere*, 2010, 78, 209–215.
- 63 S. B. Lovern, H. A. Owen and R. Klaper, Electron microscopy of gold nanoparticle intake in the gut of *Daphnia magna*, *Nanotoxicology*, 2008, 2, 43–48.
- 64 P. Rosenkranz, Q. Chaudhry, V. Stone and T. F. Fernandes, A comparison of nanoparticle and fine particle uptake by *Daphnia magna*, *Environ. Toxicol. Chem.*, 2009, 28, 2142–2149.
- 65 D. Ebert, *Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]*, Chapter 2: Introduction to *Daphnia Biology*, Bethesda (MD): National Center for Biotechnology Information (US), 2005, Available from: <http://www.ncbi.nlm.nih.gov/books/NBK2042/>.
- 66 S. B. Lovern, J. R. Strickler and R. Klaper, Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle dispersions (titanium dioxide, nano-C₆₀, and C₆₀H_xC₇₀H_x), *Environ. Sci. Technol.*, 2007, 41, 4465–4470.
- 67 A. Okamoto, M. Yamamuro and N. Tatarazako, Acute toxicity of 50 metals to *Daphnia magna*, *J. Appl. Toxicol.*, 2015, 35, 824–830.
- 68 A. Dabrunz, L. Duester, C. Prasse, F. Seitz, R. Rosenfeldt, C. Schilde, G. E. Schaumann and R. Schulz, Biological surface coating and molting inhibition as mechanisms of TiO₂ nanoparticle toxicity in *Daphnia magna*, *PLoS One*, 2011, 6, e20112.
- 69 E. Kadar, P. Rooks, C. Lakey and D. A. White, The effect of engineered iron nanoparticles on growth and metabolic status of marine microalgae cultures, *Sci. Total Environ.*, 2012, 439, 8–17.
- 70 K. D. Grieger, A. Fjordbøge, N. B. Hartmann, E. Eriksson, P. L. Bjerg and A. Baun, Environmental benefits and risks of zero-valent iron nanoparticles (nZVI) for in situ remediation: risk mitigation or trade-off?, *J. Contam. Hydrol.*, 2010, 118, 165–183.

