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Ecotoxicological Assessment of Silica and Polystyrene Nanoparticles assessed by a multitrophic test battery.

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Abstract.

The acute ecotoxicity of different diameters of silica and polyethyleneimine polystyrene (PS-PEI) nanoparticles (NPs) was assessed on a test battery of aquatic organisms representing different trophic levels. *Daphnia magna*, *Thamnocephalus platyurus*, *Pseudokirchneriella subcapitata* and *Vibrio fischeri*, were employed in a series of standard acute ecotoxicity tests and work was complemented with two cytotoxicological end points on a rainbow trout gonadal cell line (RTG-2). Physico-chemical characterization of the NPs was performed in the different test media employed, using dynamic light scattering (DLS) and zeta potentiometry. In contrast to silica NPs exposure, for which no effect was observed for concentrations up to 1000 μg ml^{-1} for all *in vivo* aquatic organisms tested, significant toxicity was detected after exposure to PS-PEI NPs at concentrations from 0.40 μg ml^{-1} to 416.5 μg ml^{-1}. Differing sensitivities for each NP diameter for the different organisms was observed as: *P. subcapitata* $\geq$ *D. magna* > *T. platyurus* > *V. fischeri.* The effects observed were dependent in some cases on the NP size, a higher effect being observed for the larger NPs. Finally, cytotoxicity studies showed an effect at the highest concentrations for both sets of NPs which was greater in the case of the PS-PEI NPs. However, as agglomeration and sedimentation of the nanoparticles was observed at these concentrations, the cytotoxicity studies were found not to be a reliable ecotoxicity test model.

Keywords: Nanopolystyrene; Silica Nanoparticles; Ecotoxicity; Cytotoxicity.

1 Introduction.

The development of materials and products at the nanoscale has become a major investment area on a global level. Nanotechnology is largely based on these materials, generally defined as nanomaterials (NMs), which, for regulatory purposes, have been recently defined by the European Commission (EC) as any natural, incidental, or manufactured particulate material which is in the unbounded, aggregated or agglomerated form and with at least a 50 % of the particles in the number size distribution that has at least one dimension in the size between 1 and 100 nanometers (nm) (EC 2011). NPs fall within this definition, but to be more specific, NPs are defined as particulate materials with three dimensions of the order of 100 nm or less (Loevestam et al. 2010).

NMs often exhibit enhanced or different properties when compared with the bulk material due to their extremely small size, consequent high specific surface area, surface energy and other factors such as larger proportions of under co-ordinated bonds and spatially constrained electronic wavefunctions (Lead and Wilkinson 2006). Certain NMs can offer, amongst others, distinct optical, electrical and magnetic properties, rendering them of great potential in a very wide range of fields and applications (Rao and Cheetham 2001). These, and other properties, make NMs very useful in technology and their use is rapidly increasing due to their applications in areas such as textiles, electronics, pharmaceutics, cosmetics and environmental remediation (Roco 2005).

NPs can be divided into natural and anthropogenic NPs. In the latter case, NPs can be formed unintentionally as a by-product, generally by combustion or formed intentionally, in which case they are termed manufactured or engineered NPs (ENPs) (Nowack and Bucheli 2007). According to 'The Project on Emerging Nanotechnologies' inventory, the production of nanotechnology-based consumer products has increased 521 % since March 2006, reaching a total of 1317 products currently on the market worldwide (2005). However, the increased use of NPs increases the likelihood of environmental exposure to NPs and poses questions as to specific NP-associated hazards. In addition, although a NP type may be characterized as nontoxic, aggregation or interaction with the exposure medium may affect their properties, mobility and hence exposure in poorly understood ways (Slaveykova and Wilkinson

2005). Concerns are thus raised by the possible release of certain novel ENPs into the environment and their potential effects on the aquatic ecosystem.

Although nanotoxicological research started in the early 1990s, research on the effects of NPs on environmentally relevant species has only emerged in the recent years, the first reports being published in 2006 (Hund-Rinke and Simon 2006). In a relatively short time, however, hundreds of studies and dozens of review papers on nanoecotoxicology have emerged (Farre *et al.* 2009; Kahru and Dubourguier 2010; Navarro *et al.* 2008). In general, nanoecotoxicologists are challenged to develop new protocols suitable for NPs, using the already large experience and toxicity data published on the evaluation of the bulk chemical as an environmental hazard (Baun *et al.* 2008; Handy *et al.* 2008; Klaine *et al.* 2008).

In this study, standard ecotoxicity tests used for bulk chemicals were explored, with slight modifications to the protocols to better suit these novel test materials. The aquatic species selected for the toxicity tests in this study, ranging from fish cell lines, algae, crustaceans and bacteria, are representative of a range of trophic levels. They are simple established tests using organisms known to be especially sensitive to a wide range of pollutants and to have a standard reproducible response to facilitate inter-laboratory comparisons. The responses of the test species to two types of model ENPs are compared in order to evaluate their suitability and relative sensitivities for NP screening. A similar test battery has been employed to characterize the responses to a series of co-polymer (Naha et al. 2009a) and dendritic polymer NPs (Naha et al. 2009b) of systematically varying structure, and thus the consistency of study design enables comparison of materials response.

The study will focus exclusively on amorphous silica NPs and polystyrene (PS) NPs. Crystalline silica NPs are known for their high toxicity *in vivo* and *in vitro* (Napierska et al. 2010) and in some cases are being used as positive controls within other NP studies (Lin et al. 2006). Crystallinity is a very important property of silica that is proportionally linked to its toxicity. Amorphous silica particles, in comparison, are considered to be relatively harmless and are therefore being produced in large quantities for a large number of applications, especially in biomedical applications and the food sector

(Wang et al. 2006). Thus, particular concern arises about their possible toxicity and a number of studies have already shown both non toxic (Barnes *et al.* 2008) and toxic effects with amorphous silica NPs (Van Hoecke et al. 2008), and specifically in the form of pulmonary inflammation upon inhalation (Rosenbruch 1992).

Synthesized from organic polymers, with the possibility to produce them in different sizes, surface charge, composition and morphology, polymeric NPs can be obtained with very different functional properties that makes them a perfect product for a wide range of applications (Nowack and Bucheli 2007). Depending on the polymer type, they can potentially be used in a range of applications, the main one being in the medical sector for drug delivery (Chan et al. 2010). PS NPs can be divided into three main groups according to their effective surface charge; cationic, anionic or neutral (unmodified) PS NPs. Their surface charge will depend on the surface coating, the most common functional groups used being NH_2- for cationic and COOH- for anionic surfaces. This allows them to pass more easily through the cell membrane, as they share a similar molecular structure to proteins, rendering them a potential tool for drug delivery. However, some studies have shown that, while the neutral and negatively charged PS NPs are considered to be nontoxic, the positively charged PS NPs induce some toxicity (Liu *et al.* 2011).

In this study, the responses of the battery of ecotoxicological test species to silica and PS nanoparticles, chosen as model compounds, are compared in order to evaluate their suitability and relative sensitivities for NP screening. Although the selected nanoparticles may not accurately represent the materials used in actual consumer products, the model systems are employed as reliable, well defined, physically and chemically model particles for demonstration studies. The results are compared to previous studies of polymeric nanoparticle systems of systematically varied physico-chemical properties. The suitability of the model NPs as positive and negative controls for NP screening is also evaluated.

2 Materials and methods.

2.1 Test compounds.

Two different sizes of plain silica NPs and green fluorescently labeled silica NPs were purchased from Kisker Biotech GmbH & Co (Germany). These are amorphous, monodisperse silica beads, of 50 nm and 100 nm nominal diameters, with a density of 2.0 g $(cm^3)^{-1}$ per particle and are supplied in 10 ml aqueous suspensions of 25 μg ml^{-1} and 50 μg ml^{-1} concentrations respectively. The excitation and emission wavelengths of the fluorescently green labeled silica NPs are 485 nm and 510 nm respectively.

PS-PEI NPs were manufactured and supplied by the Centre for BioNano Interactions (University College Dublin (UCD), Ireland). Briefly, these PS-PEI particles are synthesized from carboxylated PS NPs (also manufactured by UCD), whereby the carboxylate surface group reacts with the amine of the PEI using EDAC (N-(3-Dimethylaminopropyl)-N•-ethylcarbodiimide hydrochloride) as a dehydrating agent. Two different nominal sizes were supplied; 55 nm and 110 nm diameter, both in a stock concentration of 30 mg ml^{-1} suspended in deionised water. Phenol (CAS No. 108-95-2) and potassium dichromate (CAS No. 7778-50-9) were employed as positive reference toxicants and were purchased from Sigma-Aldrich (Ireland).

2.2 Nanoparticle characterization.

Selected physico-chemical properties of the different sizes of fluorescently labeled silica NPs and aminated PS NPs tested were characterized over time and at several representative exposure concentrations in the different media used in the assays (no organisms/cells present). The NP size, as characterized by their hydrodynamic diameter, the effective surface charge, as characterized by their zeta potential were determined, and agglomeration state monitored, using dynamic light scattering and zeta potentiometry, with the aid of a Malvern Instruments Zetasizer Nano Series (Particular Sciences, UK) operating with version 5.03 of the system's Dispersion Technology Software (DTS Nano), in order to confirm, in the case of the Silica NPs, the manufacturers' specifications, and in the case of the PS nanoparticles, to characterize their physico-chemical properties. Characterization of silica NPs was

carried out only on fluorescently labeled silica NPs as plain silica NPs are manufactured under the same conditions, and have identical technical specifications (Kisker Biotech GmbH & Co).

For each experimental replicate, samples of fluorescent silica NPs and PS-PEI NPs were freshly prepared from their stock solutions by dilution into the respective media in order to obtain concentrations of 10, 100 and 1000 μg ml^{-1} and 1, 10 and 100 μg ml^{-1} respectively. No specific sonication/shaking/stirring procedure was employed except for the cytotoxicity assays, which were bath sonicated for 30 minutes. DLS analysis was performed immediately for time = 0 hours at the different concentrations mentioned.

In order to determine whether particles sediment over time in the test media, further analysis was undertaken at the following endpoint times and the following relevant concentrations for each test in their respective media; 100 μg ml^{-1} fluorescent silica NPs and 1 μg ml^{-1} PS-PEI NPs concentration for Algal medium [AM] after 72 hours, 1000 μg ml^{-1} fluorescent silica NPs and 10 μg ml^{-1} PS-PEI NPs concentration for Thamnotox medium [TM] and for Elendt M4 *Daphnia* medium after 24 hours and 48 hours respectively and 1000 μg ml^{-1} fluorescent silica NPs and 100 μg ml^{-1} PS-PEI NPs concentration for the cell culture medium Dulbecco's modified medium nutrient mix / F-12 Ham [DMEM] after 24, 48, 72 and 96 hours exposure. It should be noted that the above prepared samples were maintained over the duration of the measurement under the same conditions (shaking/illumination/temperature) as in the exposure experiments detailed in the following sections.

Approximately 1.5 ml of the sample suspension of NPs in their respective assay media (Milli-Q water [MQ], Microtox diluent [MD], AM, TM, Elendt M4 *Daphnia* medium and DMEM) in 10x10x45 mm polystyrol/polystyrene cuvettes were inserted for DLS analysis at 20 $^{\circ}C$ for all measurements, except for the analysis in Thamnotox medium, which was set at 25 $^{\circ}C$ in order to follow the same conditions as in the toxicity assays.

The zeta potential of all particles in MQ water and the respective assay media was measured using the Zeta sizer (Malvern Instruments, UK). Approximately 3 ml of 100 μg ml^{-1} concentration of

NPs in solution were injected into a folded capillary cell for zeta potential analysis, at 20 °C for all measurements, except for the analysis in Thamnotox medium which was set at 25 °C.

2.3 Ecotoxicity tests.

The following tests were carried out, where possible, in accordance with standard guidelines. However, due to the high cost and low sample volumes of the supplied silica NPs, slight modifications to standard procedures were necessary. Any deviations from standard guidelines are described in full.

In order to establish suitable test ranges, initial range finding tests, using a series of widely spaced exposure concentrations with no replication, were conducted with the NPs and the various test species. Taking into consideration the results of the range finding tests, the definitive tests employed a concentration range (at least five concentrations and appropriate controls, as specified in the respective descriptions of the tests) in which effects were likely to occur.

2.3.1 Microtox test: *Vibrio fischeri.*

Lyophilised *Vibrio fischeri* bacteria (NRRL B-11177) and all Microtox® reagents were obtained from SDI Europe, Hampshire, UK.

The acute toxicity of 50 nm and 100 nm amorphous plain silica NPs, and 55 nm and 110 nm PS-PEI NPs to the marine bacterium *V.fischeri* was determined using the 90% basic test for aqueous extract protocol (Microtox 1998) and bioluminescence inhibition was measured at 5-, 15- and 30-min exposure time to a dilution series of concentrations ranging from 1000 μg ml^{-1} to 3 μg ml^{-1} with one replicate per test concentration. The acute toxicity data were obtained and analyzed using the MicrotoxOmni software (SDI Europe, Hampshire, UK). A basic test was also conducted for every fresh vial of bacteria prior to testing with NPs in order to ensure the viability of the test and the bacteria with the reference toxicant phenol.

2.3.2 OECD 201 Growth Inhibition of Algae: *Pseudokirchneriella subcapitata.*

Pseudokirchneriella subcapitata CCAP 278/4 were obtained from the Culture Collection of Algae and Protozoa (CCAP) Argyll, Scotland. All microalgae growth inhibition tests were conducted at 20±1 °C with continuous shaking at 100 rpm and continuous illumination (4,000 lux, cool-white fluorescence, measured with a Lux meter [Lutron Electronic LX-101]).

Assessment of the acute toxicity of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs to the freshwater algae *P. subcapitata,* was conducted in accordance with the OECD Guideline 201 (OECD 2006) with some variations. Exposure to a limit test of 100 µg ml^{-1} silica NPs concentration was conducted with 6 replicates. Similarly, exposure to 5 different concentrations, ranging from 0.1 µg ml^{-1} to 1.0 µg ml^{-1} for 55 nm PS-PEI NPs and from 0.1 µg ml^{-1} to 0.8 µg ml^{-1} of 110 nm for PS-PEI NPs, was conducted with 3 replicates per test concentration. The initial algal density of all flasks was 1×10^{4} cell ml^{-1} in a final volume of 20 ml and 6 negative controls were incorporated for each test containing only algal growth media and algal inoculum. The cell density of each replicate was measured after 72 h using a Neubauer Improved (Bright-Line) chamber (Brand, Germany) and growth was quantified from measurements of the algal biomass as a function of time. Average specific growth rate (µ) and percentage inhibition of average specific growth rate (%Ir) relative to controls were calculated and the Median Effective Concentration (EC_{50}) was determined. The pH of the controls and the highest NP concentrations were measured at the start and end of the experiment (Table 4. Supplementary information)

Potassium dichromate was employed as a positive control in accordance with the OECD Guideline to ensure validity of the test method and the EC_{50} calculated and compared to the expected EC_{50} according to the literature (Nyholm 1990).

2.3.3 Thamnotox test™: *Thamnocephalus platyurus.*

This toxicity test was purchased in kit form from SDI Europe (Hampshire, UK) and the test was performed according to manufacturer's instructions (Thamnotox 1995). Briefly, the test is a 24 h Median Lethal Concentration (LC_{50}) bioassay, which is performed in a 24-well test plate using instar II–

III larvae of the shrimp *Thamnocephalus platyurus*, which are hatched from cysts. Upon hatching, 10 shrimp per well were exposed to a range of 5 concentrations in triplicate in standard freshwater medium, ranging from 0.1 to 1000 µg ml^{-1} in the case of 50 nm and 100 nm amorphous plain silica and fluorescently labeled silica NPs, and from 3 to 20 µg ml^{-1} for 55 nm PS-PEI NPs and from 2 to 15 µg ml^{-1} for 110 nm PS-PEI NPs. These were incubated at 25 °C for 24 h in the dark. The test endpoint was mortality (no observed movement after 15 seconds and gentle agitation). At test termination, the number of dead shrimp at each concentration was recorded and the respective LC_{50} was determined.

2.3.4 OECD 202 *Daphnia magna* immobilisation test.

Acute toxicity immobilisation tests were performed on each of the NPs in accordance with OECD Guideline 202 (OECD 2004). *Daphnia magna* were kindly supplied by Shannon Aquatic Toxicity Laboratory and cultured in static conditions at 20±1 °C and under a 16 h / 8 h light / dark photoperiod for all exposures. Acute toxicity tests were performed on *D. magna* neonates that were less than 24 h old. A control and five different exposure concentrations of 0.1, 1.0, 10, 100 and 1000 µg ml^{-1} for 50 nm and 100 nm fluorescently labeled silica and plain silica NPs and 0.33, 1.0, 1.5, 2.0, and 3.3 µg ml^{-1} for 55 nm and 110 nm PS-PEI NPs were used. Four replicates were tested for each test concentration and control and five neonates were used in each replicate. There was no feeding during the tests. Immobilisation (no independent movement after gentle agitation of the test liquid for 15 s) was determined visually and recorded after 24 h and 48 h at each concentration and the respective EC_{50} values were determined. The pH of the controls and the highest NP concentrations were measured at the start and end of the experiment (Table 4. Supplementary information)

2.3.5 Cell Culture and cytotoxicity assays.

An established fish cell line was used for cytotoxicity testing. RTG-2 cells (Catalogue no. 90102529), a rainbow trout gonadal cell line, were obtained from the European Collection of Cell Cultures (Salisbury, UK). These were maintained in DMEM supplemented with 10% fetal bovine serum

(FBS) and 45 IU ml^{-1} penicillin, 45 mg ml^{-1} streptomycin, 25 mM HEPES and 1% non-essential amino acids. Cultures were maintained in a refrigerated incubator (Leec, Nottingham, UK) at 20 °C under normoxic atmosphere.

For cytotoxicity assays, RTG-2 cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 2 x 10^5 cells ml^{-1}, 1.8 x 10^5 cells ml^{-1}, 1.6 x 10^5 cells ml^{-1} and 1.6 x 10^5 cells ml^{-1} in DMEM for 24, 48, 72 and 96 hours, respectively. These seeding densities were found to be optimal to achieve 80% confluency at the end of each respective exposure period. After 24 h of cell attachment, plates were washed with 100 µl/well phosphate buffered saline (PBS) and the cells were treated with increasing concentrations of 50 nm and 100 nm fluorescently labeled silica NPs up to 1000 µg ml^{-1} and increasing concentrations of 55 nm and 110 nm PS-PEI NPs up to 200 µg ml^{-1}, both types of NP suspensions having been previously placed in a sonicating bath for 30 min approximately. Cells were maintained at 20 °C under normoxic atmosphere. Six replicate wells were used for each control and test concentration per microplate. The Alamar Blue (AB) assay, employed to assess the metabolic activity, and the Neutral Red (NR) assay, employed for the assessment of membrane function and lysosomal activity, were subsequently conducted in the same plate following the methodology as described by Davoren and Fogarty (Davoren and Fogarty 2006). Interference of the assays was checked following the protocol described by Casey *et al.* (Casey *et al.* 2007) and no interferences between the NPs and the colorimetric assays were observed (Figure 6. Supplementary information). Rather than a decrease in fluorescence, an increase in fluorescence is observed, and therefore any observed toxicity result (manifest as a decrease in fluorescence) is not the result of interference. It should be noted that the protocol of Casey *et al.* over estimates the result of the possible interactions, assuming that the particles are not washed away after exposure (in both AB and NR assays, cells are washed with PBS before the addition of the dye and the actual measurement). Any residual NP concentrations during the cytotoxicity measurement are expected to be significantly lower.

2.3.6 Statistical Analysis.

All experiments were conducted in at least triplicate (three independent experiments). Ecotoxicity was expressed as mean percentage inhibition in the case of Microtox ® (inhibition of bioluminescence), *D. magna* (immobilisation) and percentage mortality for the *T. platyurus* assay. Fluorescence (AB/NR assays) as fluorescent units (FUs) was quantified using a microplate reader (TECAN GENios, Grödig, Austria). Raw data from cell cytotoxicity assays were collated and analyzed using Microsoft Excel ® (Microsoft Corporation, Redmond, WA). Cytotoxicity was expressed as mean percentage relative to the unexposed control ± standard error of the mean (SEM), which was calculated using the formula [(mean experimental data/mean control data) × 100]. Control values were set at 100% cell viability. Statistical analyses were carried out using a one-way analyses of variance (ANOVA) followed by Dunnett's multiple comparison test. Cytotoxicity data was fitted to a sigmoidal curve and a four parameter logistic model used to calculate the EC_{50} values. This analysis was performed using Xlfit3™ a curve fitting add-in for Microsoft® Excel (ID Business Solutions, UK).

3 Results.

3.1 Characterization of particles.

3.1.1 Particle size measurement.

The average particle sizes, as characterized by their hydrodynamic diameter, of fluorescently labeled silica NPs and PS-PEI NPs in the different test media before exposure to the organism (time= 0 hours) and as a function of concentration, are shown in Table 1. For comparison, and in order to determine whether particles sediment in the test media over time, Table 2 shows the mean intensity distribution after different time exposures of the particles in different test media. Errors indicate the standard deviation over six independent measurements.

Particle size measurement (DLS) results in Table 1 and Table 2 showed no significant differences in the diameter distribution of the particles between the different media over the duration of the tests and concentrations, except in the case of the cell culture medium used for the cytotoxicity assays, shown in Table 1. In all but the cell culture medium, the distributions were quite monodisperse, with low Polydispersity Index (PdI) values in the range between 0.00 and 0.30 (Table1 and Table 2. Supplementary information), the particle size and size distributions were observed to be independent of concentration over the range studied, although a slight increase in the size distribution and PdI values were observed at the lower concentrations of 1 μg ml^{-1} in the case of the PS-PEI NPs, indicating some instability of the particles at low concentrations. For the silica NPs, the measured values were found to be consistent with the nominal values of 50 nm and 100 nm. In the case of the PS-PEI NPs, the values were found to be consistently higher than the nominal values. The nominal values refer however to the PS core, the hydrodynamic radius of which is increased by the PEI coating. Throughout the manuscript, the nominal values of particle size will continue to be employed for simplicity of nomenclature.

In the cell culture medium, the measurement registered particles as large as 200 nm and 3 μm, increasing with concentration in the case of the fluorescently labeled silica NPs and PS-PEI NPs respectively. The size was seen to be significantly increased compared to the other media, and the increase in size concentration dependent. Similar increases of apparent NP size in cell culture medium

have been observed by others, and have been attributed to interaction with the cell culture medium and/or NP aggregation/agglomeration (Rabolli et al. 2010).

In contrast to the other test culture media, the particle size distribution was seen to be unstable over time in the RTG-2 cell culture medium and sedimentation of the NPs at the highest concentrations was observed (Figure 1 and Figure 2. Supplementary information). Exposure concentrations in the rest of the assays and at lower concentrations in the cell culture media are assumed to be constant throughout the duration of the experiment. In the case of 1000 μg ml^{-1} exposure of the 50 nm silica NPs to RTG-2 cells, the initial mean of ~200 nm increased to ~300 nm after 24 hours, whereas over extended exposure periods the particle size distribution was identical to that of the unexposed control medium, as shown in Figure 1. The behavior is consistent with an initial adsorption of media components on the surface of the NPs, followed by precipitation. In the case of the 100 nm silica particles, a similar behavior is observed, although less pronounced (Figure 3. Supplementary information). In the case of PS-PEI NPs, the 55 nm PS-PEI particles show a very similar behavior as that exhibited by the 50 nm silica NPs (Figure 4. Supplementary information), whereas the 110 nm PS-PEI NPs appear to have sedimented within 24hours (Figure 5. Supplementary information).

3.1.2 Zeta potentiometry.

Zeta potentiometry of fluorescent silica NPs and PS-PEI NPs was also carried out in all media at 100 μg ml^{-1} concentration. Figure 2 summarizes the results for all zeta potential measurements. The zeta potential is derived from the electrophoretic mobility, values of which are listed in Table 3. Supplementary information.

Negative zeta potentials were obtained for silica NPs, the 100 nm silica NPs exhibiting a zeta potential consistently almost twice that of the 50 nm particles. In AM, a slight decrease in the zeta potential was observed when compared to the values in MQ. In TM, a greater reduction was observed, probably due to the salts present in the standard freshwater media. In cell culture medium and MD, the

zeta potential was reduced to a greater extent, leading to zeta potential values lower than -10 mV for both sizes NP.

Positive zeta potentials of around 60 mV were obtained for PS-PEI NPs, due to the cationic coating of the NP. Both sizes of the particles exhibited similar zeta potentials, as expected, as the interactions of the particles with their environment are governed by the surface rather than the core. In DM, a slight decrease in the zeta potential was observed when compared to the values in MQ. Similar values were observed in AM, and TM with lower zeta potential values, but these were still > 30 mV, indicating the dispersion is stable and unlikely to experience agglomeration. In MD, however, a larger reduction is observed, yielding zeta potential values < 30 mV, which could lead to agglomeration of the particles and instability, making them less bioavailable in the test assay. This is due to charge neutralization with the salts present in the media; as *V. fischeri* is a marine bacterium, the MD has a high ionic strength which is bound to affect the stability of the particle. In cell culture medium, the zeta potential is reduced to a greater degree, to the point of obtaining negative surface charge values around 5 - 7 mV, indicating that the coating from the proteins (negatively charged) in the media dominates the particle surface.

In summary, the reductions of the zeta potential were potentially due to interactions with the molecular constituents of the medium in the case of the cell culture medium (Sager et al. 2007) and charge neutralization due to the salts present in the other media. However, with the exception of the cell culture medium, the reduced zeta potentials did not appear to influence the quality of the dispersion, as there was no indication of agglomeration in Table 1.

3.2 Ecotoxicity.

The two different sets of NPs were tested on several standard and representative ecotoxicity tests for comparison. Different responses were obtained for the different particles and results were analyzed and discussed according to the particle characteristics. Testing of the reference chemicals, phenol, and potassium dichromate, was carried out in tandem with the NPs to ensure the validity of each test method.

End points of all reference toxicity tests were within those stipulated in each respective standard guideline for the case of phenol and *V. fischeri* (Microtox 1998), or reported in other previous studies for the case of potassium dichromate and *P. subcapitata* (Nyholm 1990). Consistent results were achieved for each test control in accordance with the criteria for validity of each test guideline. A summary on the results with the EC_{50} values of silica and PS-PEI NPs on the different test models are shown in Table 3.

Plain and fluorescently labeled silica NPs showed no significant toxicity in any of the acute ecotoxicity tests performed on the different organisms for both diameters. Such a response may be expected as amorphous silica NPs are known for their low toxicity (Barnes *et al.* 2008; Rabolli *et al.* 2010), indicating their suitability as a good negative control for NP exposure. In the case of the cytotoxicity testing, both assays indicate a low dose and exposure time dependent response as shown in Figure 3, a slightly larger effect being observed for the 50 nm than the 100 nm silica NPs in both assays. The AB assay also showed a larger effect than the NR assay and the response was larger at the highest concentrations (1000 μg ml^{-1}).

In contrast, both NP diameters of PS-PEI NPs showed a significant toxic response in most of the acute toxicity tests performed, except for the microtox test, as shown in Table 3, where a much weaker effect was observed, probably due to particle agglomeration as discussed in the characterization section 3.1. The effects observed with PS-PEI NPs were dependent on the NP size in some assays, the size effect being statistically significant when an ANOVA of two factors was performed on the algal, thamnotox and *D. magna* results ($p<0.05$). In all cases, a greater effect from the 110 nm particles was observed when compared to the 55 nm particles, indicating that the effect observed, could not only be due to the reactive functional groups on the NP coating, but as a possible core size effect also, as per unit mass/volume concentration, the 55 nm particles present a higher degree of surface functionalisation than the 110 nm particles. A difference in the sensitivity for both NP diameters with the different organisms is observed as follows: *P. subcapitata* $\geq$ *D. magna* > *T. platyurus* > RTG-2 > *V. fischeri.* The difference in observed sensitivity was found to be in accordance with studies using other NPs found

in the literature, where algae and crustaceans (Daphnids) were the most sensitive organisms in aquatic exposure to NPs (Kahru and Dubourguier 2010). In fact, although algae were shown to be the most sensitive organism in this study, *D. magna* also showed a strong sensitivity to NP exposure, exhibiting almost equal but slightly lower EC_{50} values than those of the algal species. Cytotoxicity results expressed as EC_{50} values in Figure 4 showed a higher dose and exposure time dependent response than the silica particles in both assays, again showing a greater effect at the higher concentrations (100 - 200 μg ml^{-1}). In both assays AB and NR, and in general over the different exposure times, the 110 nm particles showed a slightly higher cytotoxicity than the 55 nm particles.

4 Discussion.

The ecotoxicity tests employed and shown here are validated and widely used standardized short-term methods for estimating the acute and chronic toxicity of chemical toxicants to bacteria, algae, invertebrates and fish. These require a specific media composition and light/dark conditions in order to simulate, in a closest possible way, realistic environmental conditions. The study shows how very different responses were obtained for the different ecotoxicity tests depending on the different biological models, as each of them will possess different cellular properties. Although the battery of tests employed was focused mainly on freshwater species, the microtox test with the marine bacteria *V. fischeri* has previously been shown to provide a good correlation with other species for a large number of chemical toxicants (Kaiser 1998). It is also considered to play an important role, as it sits at the base of most food webs and provides essential ecological and biochemical services, making it a good starting point to any ecotoxicity test. Furthermore, it is a very simple, fast, robust and cost-effective assay (Parvez et al. 2006).

In general, and according to previous ecotoxicity studies testing a broad range of chemicals, when toxicity is observed, fish is expected to be the least sensitive trophic level when compared to algae and *Daphnia*, and algae is expected to be the most sensitive trophic level when compared to fish and *Daphnia* (Weyers *et al.* 2000). The results presented here are consistent with such a conclusion.

Amorphous silica NPs were shown not to exhibit any toxic effect in most of the tests performed for any of the wide range of concentrations employed, except in the cytotoxicity tests, in which a weak dose and exposure-time dependent response was observed at the highest concentrations. These generally low responses have been shown to be in accordance with other ecotoxicity studies using engineered amorphous silica NPs, where little or no toxicity was observed in the tests employed (Barnes *et al.* 2008; Shapero *et al.* 2011; Van Hoecke *et al.* 2008). Thus, the lack of toxicity observed to date with amorphous silica NPs, suggests that, in the different standard toxicity methods, this could generally be used as a good negative NP control, except in the case of the cytotoxicity assays which themselves are shown not to be suitable to NP testing.

In contrast, PS-PEI NPs exhibited a strong toxic response for most of the tests employed, except for the microtox test. In fact, the EC_{50} values determined in this study indicate a stronger toxic response compared to other ecotoxicological studies reported with co-polymers (Naha et al. 2009a) and dendritic polymers (Naha et al. 2009b). *D. magna* was shown to be one of the most sensitive species of the test battery employed in both previous studies, exhibiting the lowest EC_{50} values of ~ 60 μg ml^{-1} for N-isopropylacrylamide (NIPAM)/*N*-tert-butylacrylamide (BAM) 50:50 co-polymer NPs (Naha et al. 2009a) and ~ 8 μg ml^{-1} (0.13 μM) for polyamidoamine (PAMAM) dendrimers of generation G-6 (Naha et al. 2009b). These findings are in agreement with those of the current study, where, although in our study *D. magna* was not the most sensitive specie, the EC_{50} values for *D. magna* were very close to those of the most sensitive, the algal test. For the PS-PEI NPs tested in the current study, the EC_{50} values for *D. magna* were ~ 0.7 μg ml^{-1}, indicating a greater toxicity than that observed in the co-polymers and dendritic polymer work. A similar trend is observed in terms of, not only sensitivity of species, but also degree of toxicity to PS-PEI NPs when comparing to the work with the PAMAM dendrimers. This is understandable, as PS-PEI NPs share a similar surface chemical structure to PAMAM dendrimers, suggesting that the surface amino groups play an important role in the toxic effects observed. Furthermore, and similar to the PAMAM dendrimer work, a NP size dependence effect is also observed on the algal, thamnotox and *daphnia* assays, the larger particle of 110 nm diameter size exhibiting a greater effect than the 55 nm diameter size, therefore showing a clear dependence on the physico-chemical properties of the NP regardless of their mode of action. Moreover, the results obtained for PS-PEI NPs are consistent with what was expected for polycationic polymers as they are known to induce the formation of nanoscale holes in the lipid bilayer and consequently enhance permeabilization of the cell membrane (Hong *et al.* 2006). Once internalized, they have been demonstrated to cause oxidative stress by the generation of elevated levels of reactive oxygen species (Mukherjee et al. 2010). Their mode of toxic action is thus specifically due to their surface activity. This also shows the importance of the surface charge of the particle, as a cationic surface would enable the particle to interact with the cell membrane more easily due to their similar molecular structure to proteins, hence, promoting the cell

uptake of the NPs (Nel *et al.* 2009). The results support the proposal that aminated polystyrene particles may be, where suitable for NP testing, appropriate positive controls for nano (eco) toxicity testing.

Finally, although our cytotoxicity results showed a significant effect at the highest concentrations for both particles, our DLS results over time at those concentrations suggested precipitation of the particles with the consequent depletion of the medium as they are coated on the particle surface. At concentrations of 1000 μg ml^{-1} for the silica particles, and 200 μg ml^{-1} for the PS-PEI NPs, the particle size has increased to over 200 nm, and 3 μm respectively at zero exposure time (Table 1), indicative of adsorption of components of the media onto the NP surface, and/or considerable particle aggregation/agglomeration at these concentrations in the fish cell culture medium. Furthermore, the aggregated NPs are seen to sediment out from the dispersion. The process may result in significant depletion of the medium, leading to an indirect toxic effect as observed for example in the case of exposure of mammalian cell lines to carbon nanotubes (Casey et al. 2008) and, furthermore, any interaction of such aggregates with the cells cannot be considered as a NP effect. Using NR, a viability assay, little or no response was observed below these concentrations indicating no NP induced cell death. AB is also a monitor of proliferative capacity and the reduction in the assay response at low doses may be a reduction of proliferative capacity due to medium depletion, as previously observed using both colorometric and clonogenic assays (Herzog et al. 2007). Therefore, our results show that fish cell lines are not a good reliable model for cytotoxicity testing of NPs, especially when the NP is unstable in solution.

In summary, in this manuscript we described the comparative toxicity of two different types of NPs with extremely different responses for each type, suggesting them, where suitable for NP testing, as possible good positive and negative NP controls for PS-PEI and amorphous silica NPs respectively. The concentrations employed, were much higher than would be expected in the environment based on model predictions of NP release from consumer products (Gottschalk et al. 2009). However one of the purposes of this study was to assess the suitability of standard ecotoxicity protocols for the assessment of NP toxicity, thus concentrations employed were chosen following the guidelines in order to observe a

toxic response. Further investigations about the possibility that NPs could be transferred between the different trophic levels through exposure to food are suggested as future work, as has already been demonstrated with Quantum Dots and TiO_2 (Bouldin *et al.* 2008; Zhu *et al.* 2010).

These results thus provide a better insight into the suitability of standard toxicity protocols for NP assessment. New variations or modifications to the existing protocols should be studied and suggested in order to be able to develop in the future, new ecotoxicity protocols appropriate for NPs.

Acknowledgements.

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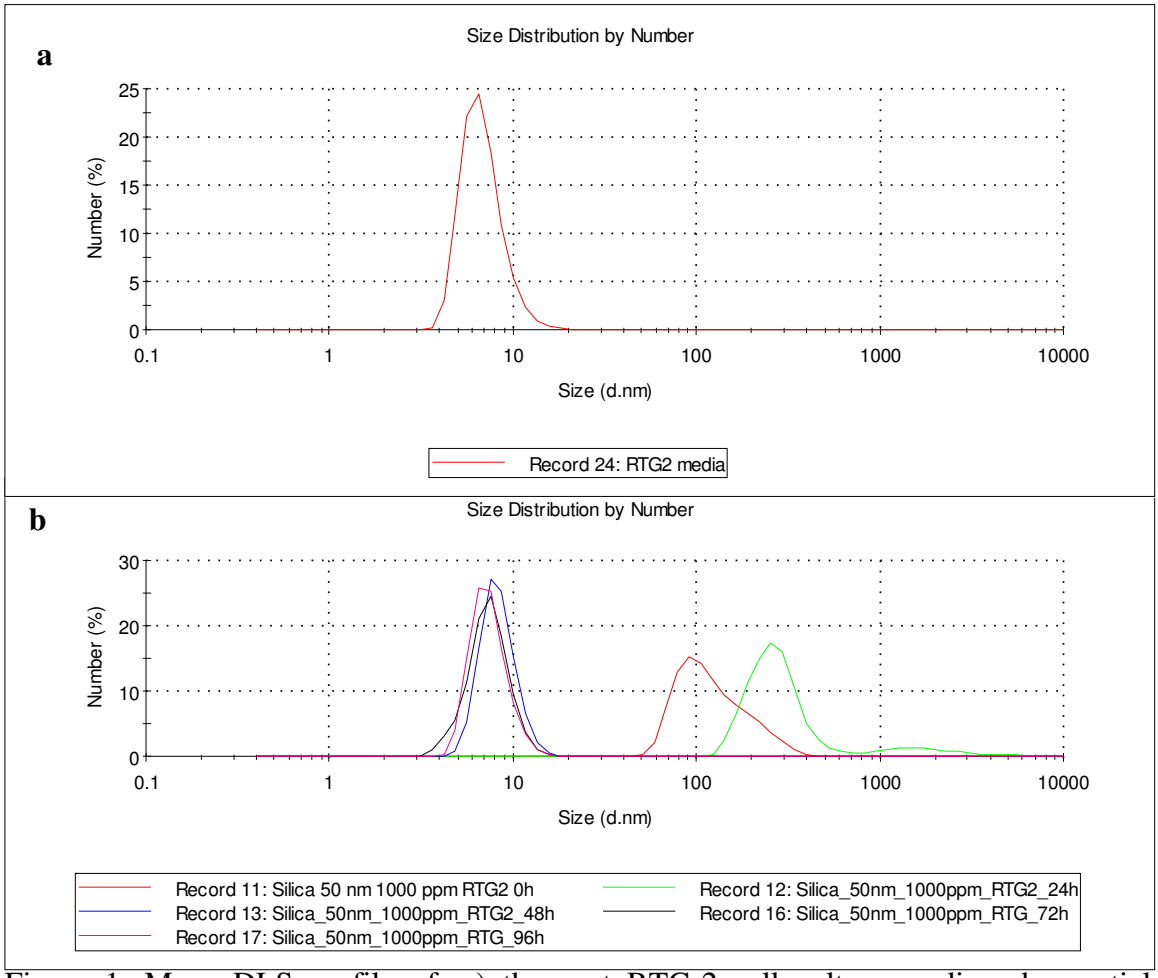

Figure 1. Mean DLS profile of: a) the neat RTG-2 cell culture medium by particle number size distribution (with no NPs added), b) particle number size distributions of 1000 µg ml^{-1} concentration of 50 nm fluorescently labelled silica NPs in RTG-2 cell culture medium after 24, 48, 72 and 96 hours exposure (n=6).

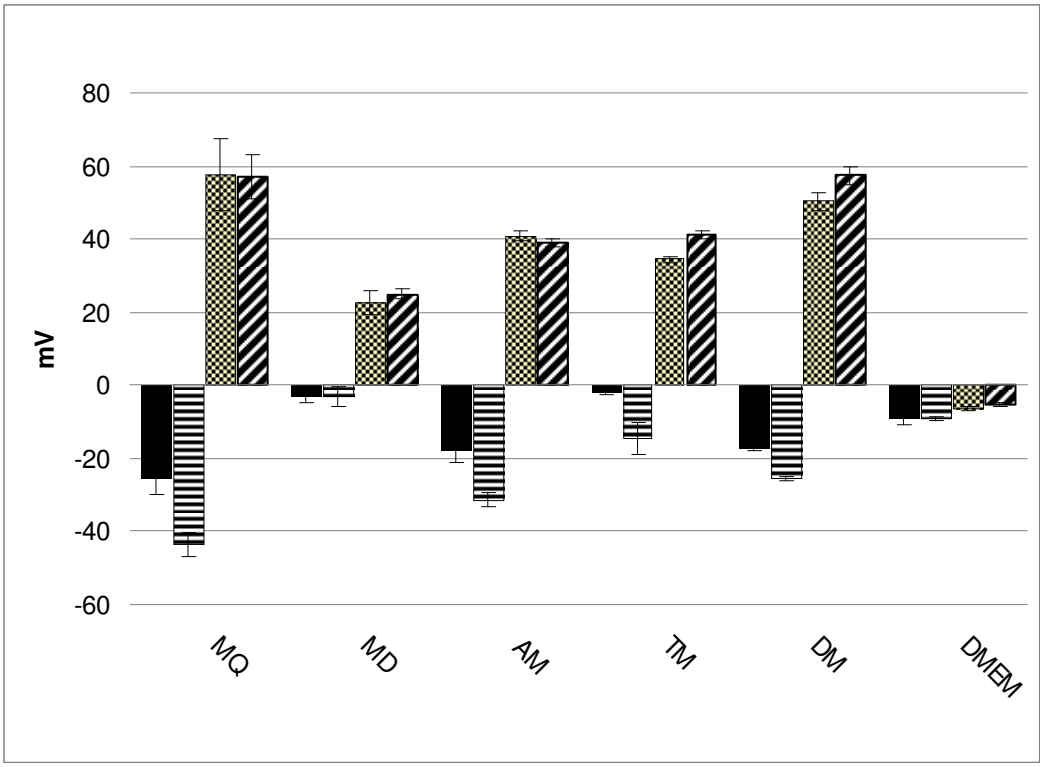

Figure 2. Zeta potentiometry measurements of 100 µg ml^{-1} concentration of 50 nm fluorescent silica NPs ( ), 100 nm fluorescent silica NPs ( ), 55 nm PS-PEI NPs ( ) and 110 nm PS-PEI NPs ( ) in Milli-Q water (MQ), Microtox Diluent (MD), Algal medium (AM), Thamnotox medium (TM), Elendt M4 *Daphnia* medium (DM), and the cell culture medium Dulbecco's modified nutrient mix / F-12 Ham (DMEM). Data presented as mean ± SD (n = 6).

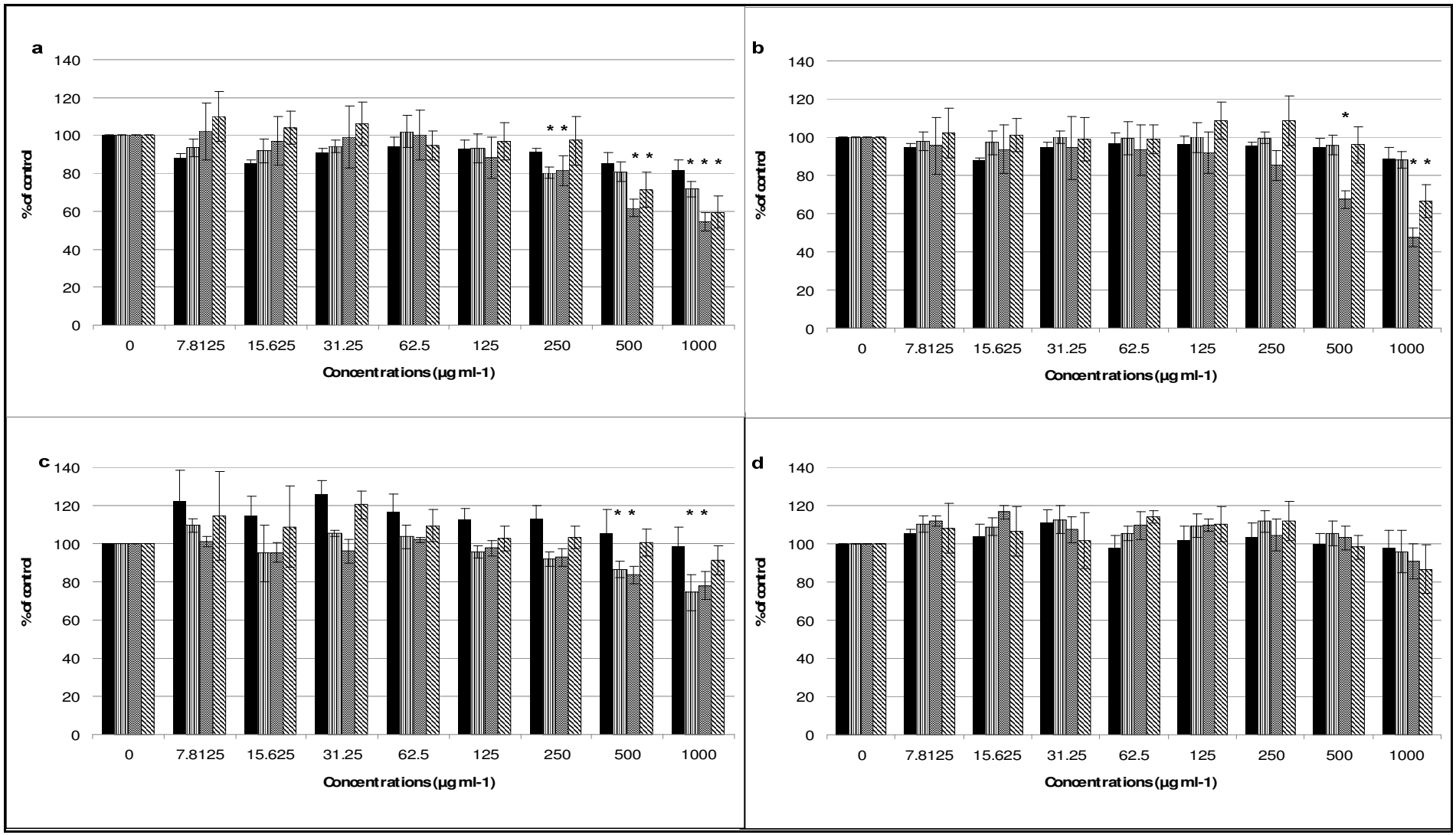

Figure 3. Cytotoxicity of 50 nm and 100 nm fluorescently labeled silica NPs to RTG-2 cells over 24 h (■), 48 h (▤), 72 h (▩) and 96 h (▨) as determined by: a) AB assay and 50 nm diameter, b) AB assay and 100 nm diameter, c) NR and 50 nm diameter and d) NR and 100 nm diameter. Data expressed as percentage of control. Data presented as mean ± SD (n = 3). (*) Statistically significant values ($P \leq 0.05$).

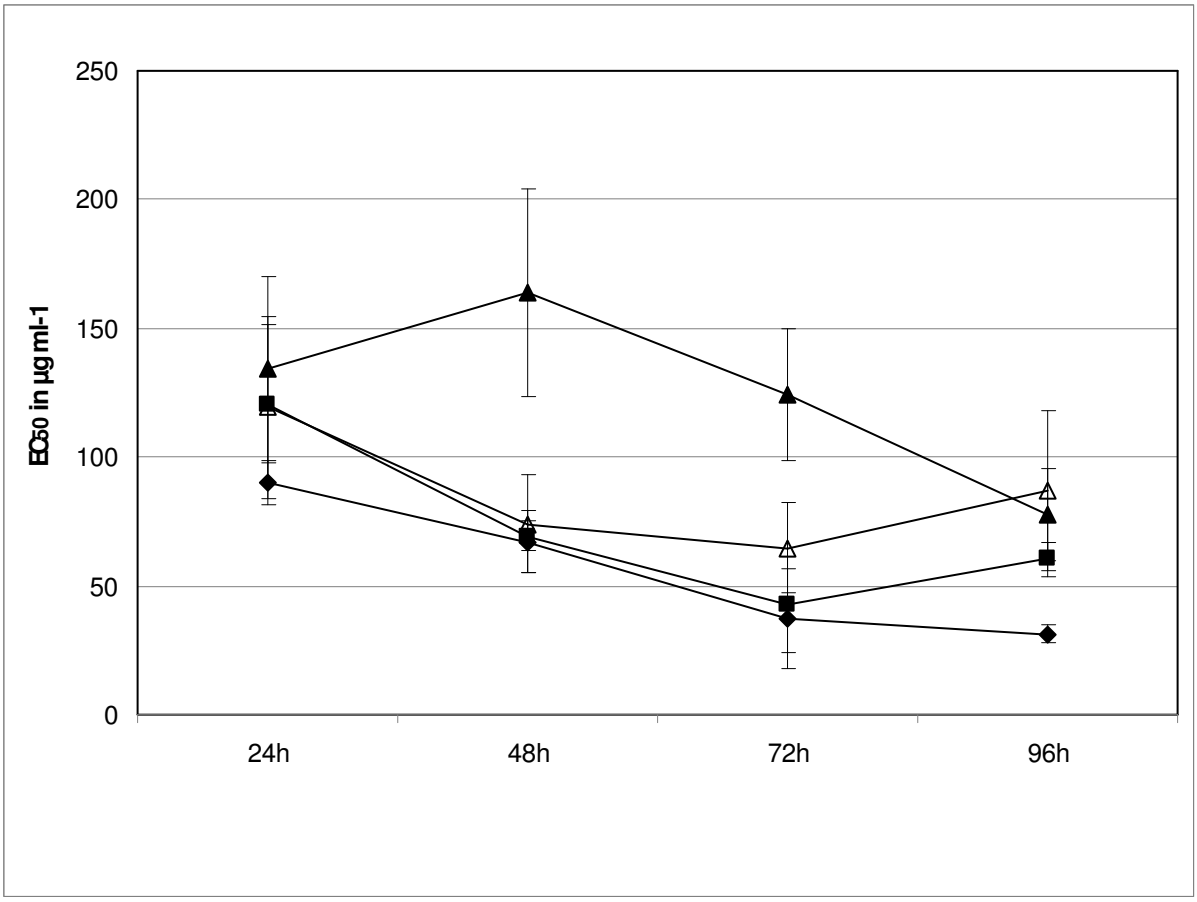

Figure 4. Cytotoxicity based on EC_{50} values over time of 55 nm PS-PEI NPs as determined by AB (-■-) and NR (-▲-) and 110 nm PS-PEI NPs as determined by AB (-♦-) and NR (-Δ-) assays. Data presented as mean ± SD (n = 3).

Table 1. Mean Zeta-average (d.nm) as measured by intensity, of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs in the different media used in the assays and their respective standard deviation (n=6) before exposure (time = 0 hours).

SILICA NPs	50 nm		100 nm		PS-PEI NPs	55 nm		110 nm	
	Z-Ave (d.nm)	STDEV	Z-Ave (d.nm)	STDEV		Z-Ave (d.nm)	STDEV	Z-Ave (d.nm)	STDEV
MQ 10 µg ml^{-1}	49.73	0.70	89.93	1.46	**MQ 1 µg ml^{-1}**	141.20	2.14	173.33	1.17
MQ 100 µg ml^{-1}	49.40	0.30	91.04	1.51	**MQ 10 µg ml^{-1}**	136.40	0.37	165.63	1.63
MQ 1000 µg ml^{-1}	51.35	0.42	93.17	0.81	**MQ 100 µg ml^{-1}**	139.55	1.18	169.38	1.63
MD 10 µg ml^{-1}	70.89	0.54	111.60	1.60	**MD 1 µg ml^{-1}**	152.42	0.99	241.05	9.21
MD 100 µg ml^{-1}	52.99	0.58	95.02	0.95	**MD 10 µg ml^{-1}**	123.10	1.18	163.95	4.66
MD 1000 µg ml^{-1}	52.07	0.17	93.17	0.55	**MD 100 µg ml^{-1}**	119.90	1.86	159.42	2.13
AM 10 µg ml^{-1}	54.81	1.41	89.80	1.36	**AM 1 µg ml^{-1}**	139.88	1.42	174.58	3.83
AM 100 µg ml^{-1}	49.82	0.22	87.51	1.14	**AM 10 µg ml^{-1}**	121.08	1.02	147.80	2.81
AM 1000 µg ml^{-1}	49.05	0.19	87.90	0.61	**AM 100 µg ml^{-1}**	116.63	2.28	144.03	2.76
TM 10 µg ml^{-1}	52.17	1.55	89.87	2.17	**TM 1 µg ml^{-1}**	126.87	2.49	185.85	1.65
TM 100 µg ml^{-1}	50.92	1.09	91.13	1.51	**TM 10 µg ml^{-1}**	122.02	1.80	149.07	1.09
TM 1000 µg ml^{-1}	49.95	0.36	89.88	1.81	**TM 100 µg ml^{-1}**	118.90	1.23	147.30	0.82
DM 10 µg ml^{-1}	58.39	2.40	100.29	4.50	**DM 1 µg ml^{-1}**	161.00	5.01	133.70	6.85
DM 100 µg ml^{-1}	63.52	4.72	89.28	0.96	**DM 10 µg ml^{-1}**	124.78	0.94	150.90	1.03
DM 1000 µg ml^{-1}	49.39	0.64	88.30	0.80	**DM 100 µg ml^{-1}**	122.75	0.39	205.35	5.06
DMEM 10 µg ml^{-1}	147.65	9.44	190.13	10.49	**DMEM 1 µg ml^{-1}**	117.51	28.82	267.02	157.67
DMEM 100 µg ml^{-1}	182.55	3.00	208.90	3.13	**DMEM 10 µg ml^{-1}**	455.35	40.79	301.98	8.40
DMEM 1000 µg ml^{-1}	204.18	3.27	214.00	2.17	**DMEM 100 µg ml^{-1}**	3072.83	864.09	3001.33	1170.41

Table 2. Mean Zeta-average (d.nm) as measured by intensity, of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs after different time exposures to different media used in the assays and their respective standard deviation (n=6).

Time exposure	SILICA NPs	50 nm		100 nm		PS-PEI NPs	55 nm		110 nm	
		Z-Ave (d.nm)	STDEV	Z-Ave (d.nm)	STDEV		Z-Ave (d.nm)	STDEV	Z-Ave (d.nm)	STDEV
72 h	**AM 100 µg ml^{-1}**	49.89	0.21	88.41	0.39	**AM 1 µg ml^{-1}**	154.54	15.84	152.43	1.12
24 h	**TM 1000 µg ml^{-1}**	49.37	0.72	89.58	1.33	**TM 10 µg ml^{-1}**	120.65	0.91	149.93	2.14
48 h	**DM 1000 µg ml^{-1}**	48.69	0.31	88.92	0.52	**DM 10 µg ml^{-1}**	119.73	1.20	148.87	0.66

Table 3. Summary of EC_{50} values for 50 nm and 100 nm fluorescently labeled and plain silica NPs and 55 nm and 110 nm PS-PEI NPs front different test models. Data presented as mean $\pm$ SD (n = 3).

Test models	**Silica 50 nm**	**Silica 100 nm**	**Test models**	**PS-PEI 55 nm**		**PS-PEI 110 nm**	
	EC_{50} (µg ml^{-1})	**EC_{50} (µg ml^{-1})**		**EC_{50} (µg ml^{-1})**	**STDEV**	**EC_{50} (µg ml^{-1})**	**STDEV**
***V. fischeri* (30 min)**	> 1000	> 1000	***V. fischeri* (30 min)**	> 1000	---	> 1000	---
***P. subcapitata* (72 h)**	> 100	> 100	***P. subcapitata* (72 h)**	0.58	0.037	0.54	0.058
***T. platyurus* (24 h)**	> 1000	> 1000	***T. platyurus* (24 h)**	5.20	0.45	4.03	0.50
***D. magna* (48 h)**	> 1000	> 1000	***D. magna* (48 h)**	0.77	0.10	0.66	0.17
RTG-2 AB (96 h)	> 1000	> 1000	**RTG-2 AB (96 h)**	60.32	6.56	31.39	3.17
RTG-2 NR (96 h)	> 1000	> 1000	**RTG-2 NR (96 h)**	77.75	17.97	87.13	30.84

Supplementary Information

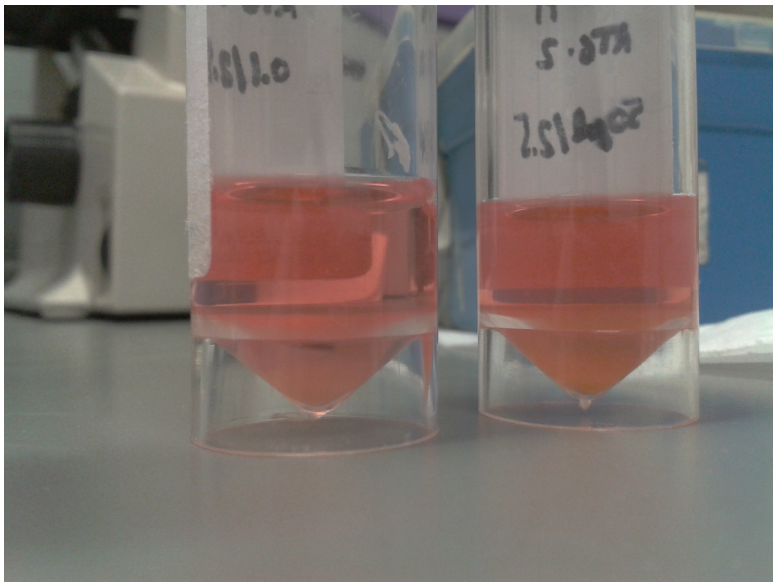

Figure 1. Digital image showing the sedimentation of 1000 µg ml^{-1} concentration of 50 nm (left) and 100 nm (right) Silica NPs after 96 h exposure in DMEM media.

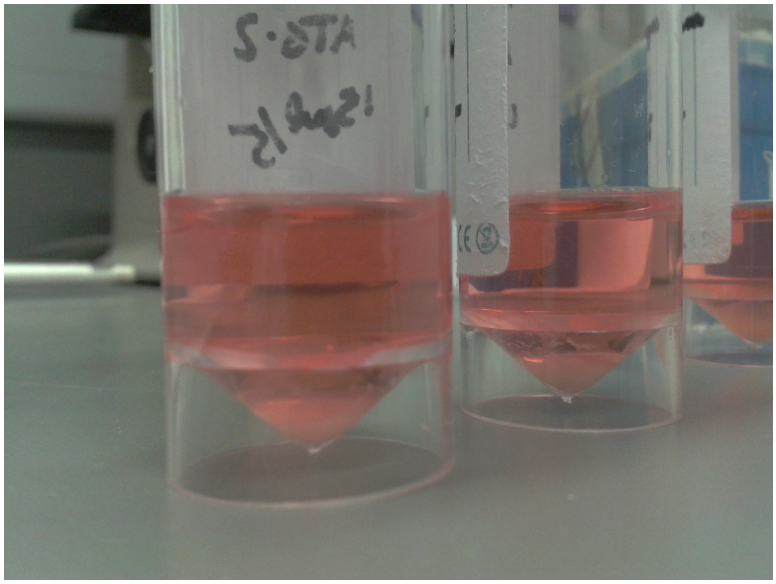

Figure 2. Digital image showing the sedimentation of 100 µg ml^{-1} concentration of 55 nm (left) and 110 nm (right) PS-PEI NPs after 96 h exposure in DMEM media.

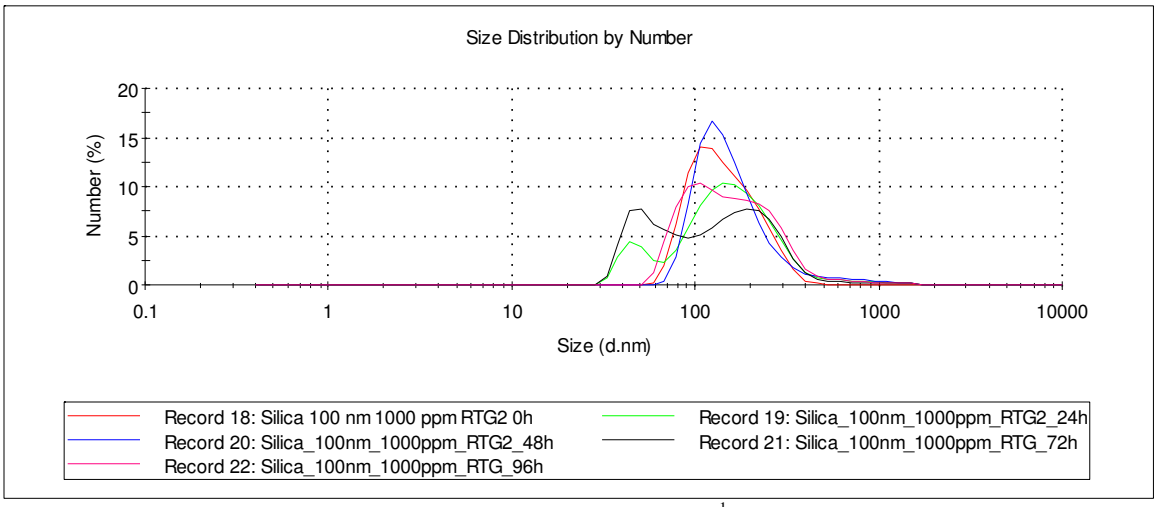

Figure 3. Particle number size distributions of 1000 µg ml^{-1} concentration of 100 nm fluorescently labelled silica NPs in RTG-2 cell culture medium after 24, 48, 72 and 96 hours exposure (n=6).

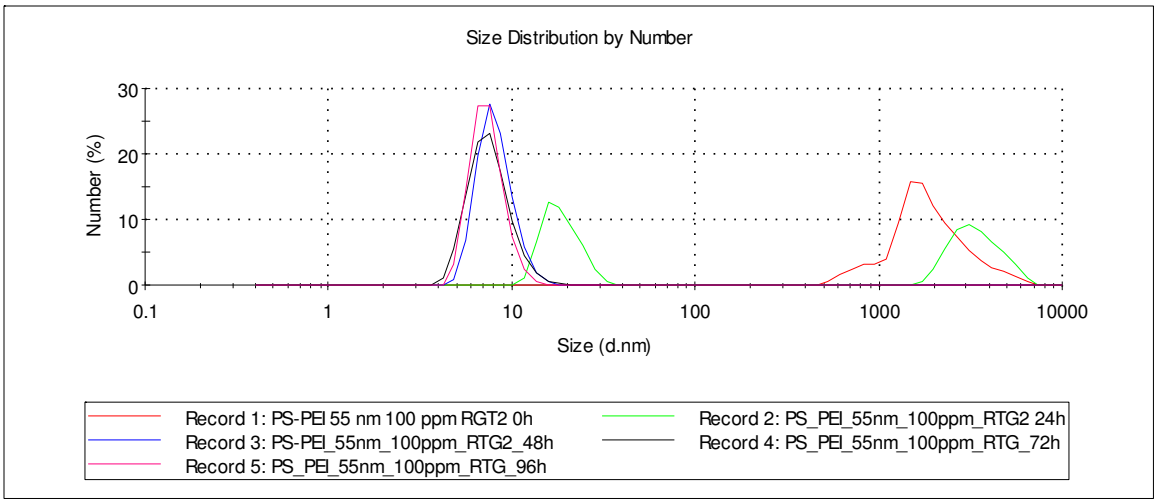

Figure 4. Particle number size distributions of 100 µg ml^{-1} concentration of 55 nm PS-PEI NPs in RTG-2 cell culture medium after 24, 48, 72 and 96 hours exposure (n=6).

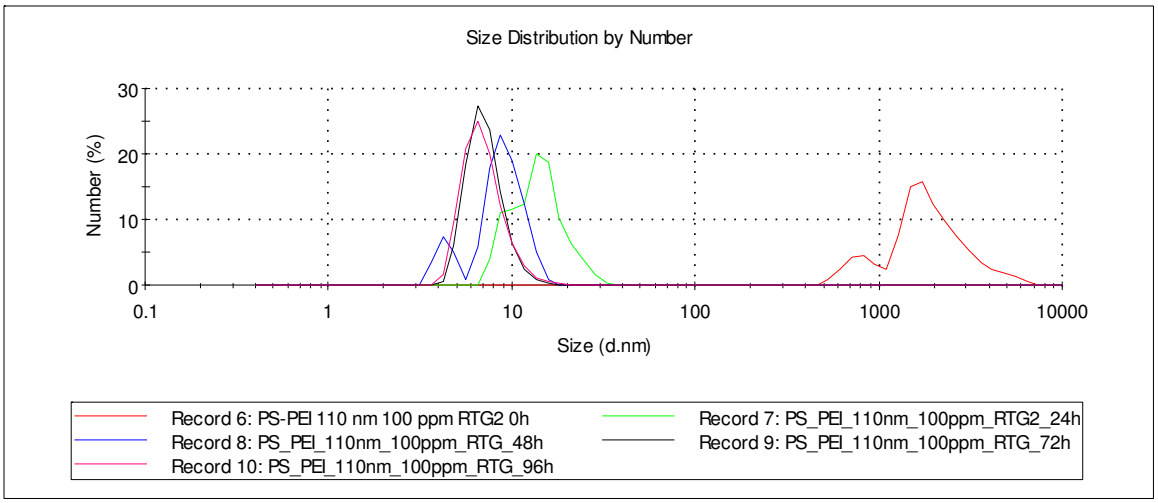

Figure 5. Particle number size distributions of 100 µg ml^{-1} concentration of 110 nm PS-PEI NPs in RTG-2 cell culture medium after 24, 48, 72 and 96 hours exposure (n=6).

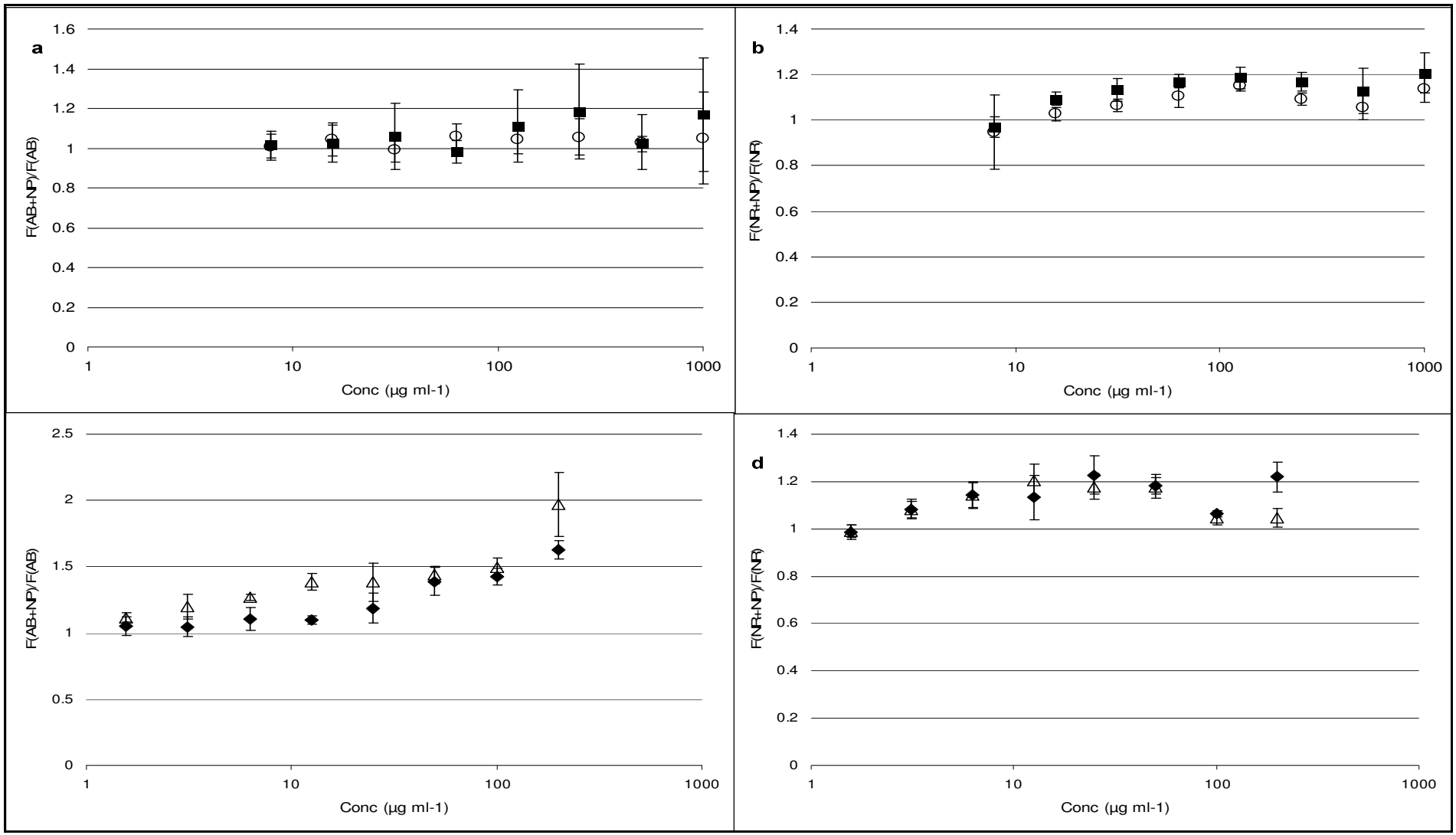

Figure 6. Plot of emission ratios for the a) AB assay and b) NR assay against 50 nm (○) and 100 nm (■) fluorescently labeled silica NPs concentration, and for the c) AB assay and d) NR assay against 55 nm (◆) and 110 nm (△) PS-PEI NPs concentration. Data presented as mean ± SD (n = 6).

Table 1. Mean Polydispersity Index (PdI) values of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs in the different media used in the assays and their respective standard deviation (n=6) before exposure (time = 0h).

SILICA NPs	50 nm		100 nm		PS-PEI NPs	55 nm		110 nm	
	PdI	STDEV	PdI	STDEV		PdI	STDEV	PdI	STDEV
MQ 10 µg ml^{-1}	0.06	0.01	0.07	0.02	MQ 1 µg ml^{-1}	0.21	0.03	0.11	0.01
MQ 100 µg ml^{-1}	0.05	0.03	0.07	0.01	MQ 10 µg ml^{-1}	0.18	0.02	0.08	0.02
MQ 1000 µg ml^{-1}	0.03	0.02	0.05	0.01	MQ 100 µg ml^{-1}	0.14	0.02	0.06	0.01
MD 10 µg ml^{-1}	0.17	0.01	0.12	0.02	MD 1 µg ml^{-1}	0.29	0.03	0.30	0.04
MD 100 µg ml^{-1}	0.07	0.01	0.08	0.01	MD 10 µg ml^{-1}	0.17	0.02	0.10	0.02
MD 1000 µg ml^{-1}	0.04	0.01	0.06	0.01	MD 100 µg ml^{-1}	0.14	0.01	0.08	0.02
AM 10 µg ml^{-1}	0.20	0.02	0.08	0.02	AM 1 µg ml^{-1}	0.28	0.02	0.21	0.03
AM 100 µg ml^{-1}	0.05	0.01	0.05	0.01	AM 10 µg ml^{-1}	0.20	0.06	0.08	0.02
AM 1000 µg ml^{-1}	0.02	0.01	0.05	0.02	AM 100 µg ml^{-1}	0.14	0.01	0.06	0.02
TM 10 µg ml^{-1}	0.12	0.10	0.06	0.01	TM 1 µg ml^{-1}	0.23	0.02	0.25	0.02
TM 100 µg ml^{-1}	0.04	0.03	0.08	0.03	TM 10 µg ml^{-1}	0.18	0.02	0.07	0.02
TM 1000 µg ml^{-1}	0.02	0.01	0.06	0.01	TM 100 µg ml^{-1}	0.14	0.01	0.06	0.03
DM 10 µg ml^{-1}	0.15	0.02	0.07	0.01	DM 1 µg ml^{-1}	0.11	0.03	0.24	0.04
DM 100 µg ml^{-1}	0.05	0.01	0.07	0.01	DM 10 µg ml^{-1}	0.16	0.01	0.06	0.02
DM 1000 µg ml^{-1}	0.02	0.01	0.05	0.01	DM 100 µg ml^{-1}	0.16	0.01	0.23	0.01
DMEM 10 µg ml^{-1}	0.51	0.07	0.86	0.01	DMEM 1 µg ml^{-1}	0.79	0.01	0.99	0.03
DMEM 100 µg ml^{-1}	0.35	0.10	0.33	0.05	DMEM 10 µg ml^{-1}	1.00	0.00	0.51	0.00
DMEM 1000 µg ml^{-1}	0.32	0.31	0.16	0.01	DMEM 100 µg ml^{-1}	0.37	0.07	0.36	0.10

Table 2. Mean Polydispersity Index (PdI) values of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs in the different media used in the assays and their respective standard deviation (n=6) after exposure.

Time exposure	SILICA NPs	50 nm		100 nm		PS-PEI NPs	55 nm		110 nm	
		PdI	STDEV	PdI	STDEV		PdI	STDEV	PdI	STDEV
72 h	**AM 100 µg ml^{-1}**	0.06	0.01	0.07	0.01	**AM 1 µg ml^{-1}**	0.32	0.03	0.11	0.03
24 h	**TM 1000 µg ml^{-1}**	0.04	0.02	0.06	0.01	**TM 10 µg ml^{-1}**	0.18	0.01	0.06	0.02
48 h	**DM 1000 µg ml^{-1}**	0.03	0.01	0.08	0.01	**DM 10 µg ml^{-1}**	0.17	0.01	0.05	0.02

Table 3. Mean electrophoretic mobility (EPM) values of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs in the different media used in the assays and their respective standard deviation (n=3).

SILICA NPs	**50 nm**		**100 nm**		**PS-PEI NPs**	**55 nm**		**110 nm**	
	EPM (µm cm V^{-1} s^{-1})	**STDEV**	**EPM (µm cm V^{-1} s^{-1})**	**STDEV**		**EPM (µm cm V^{-1} s^{-1})**	**STDEV**	**EPM (µm cm V^{-1} s^{-1})**	**STDEV**
MQ 100 µg ml^{-1}	2.00	0.35	-3.40	0.26	**MQ 100 µg ml^{-1}**	4.08	0.71	4.05	0.43
MD 100 µg ml^{-1}	-0.22	0.13	-0.26	0.21	**MD 100 µg ml^{-1}**	1.59	0.23	1.77	0.11
AM 100 µg ml^{-1}	1.39	0.28	-2.45	0.14	**AM 100 µg ml^{-1}**	2.90	0.10	2.77	0.07
TM 100 µg ml^{-1}	-0.15	0.03	-1.14	0.33	**TM 100 µg ml^{-1}**	2.70	0.07	3.23	0.09
DM 100 µg ml^{-1}	-1.35	0.04	-2.00	0.05	**DM 100 µg ml^{-1}**	3.55	0.16	4.07	0.17
DMEM 100 µg ml^{-1}	-1.25	0.18	-0.92	0.08	**DMEM 100 µg ml^{-1}**	-0.55	0.05	-0.46	0.03

Table 4. Mean pH values of 50 nm and 100 nm fluorescently labeled silica NPs and 55 nm and 110 nm PS-PEI NPs at the start and end of the assays and their respective standard deviation (n=3).

Test		Start experiment			End experiment	
	SAMPLE NAME	pH	STDEV	Time exposure	pH	STDEV
Algal growth inhibition	Control	6.37	0.02	72h	6.32	0.06
	Silica NPs 50 nm 100 µg ml^{-1}	6.32	0.03	72h	6.37	0.09
	Silica NPs 100 nm 100 µg ml^{-1}	6.3	0.02	72h	6.42	0.04
	PS-PEI NPs 55 nm 1 µg ml^{-1}	6.27	0.03	72h	6.28	0.09
	PS-PEI NPs 110 nm 0.8 µg ml^{-1}	6.19	0.03	72h	6.29	0.06
					6.44	0.12
Daphnia Immobilisation	Control	6.38	0.37	48h	6.04	0.47
	Silica NPs 50 nm 100 µg ml^{-1}	6.28	0.40	48h	6.04	0.46
	Silica NPs 100 nm 100 µg ml^{-1}	6.14	0.45	48h	6.26	0.11
	PS-PEI NPs 55 nm 3.3 µg ml^{-1}	6.57	0.40	48h	6.15	0.07
	PS-PEI NPs 110 nm 3.3 µg ml^{-1}	6.38	0.36	48h	6.32	0.06