Evaluation of the ecotoxicity of model nanoparticles

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A B S T R A C T

Since society at large became aware of the use of nanomaterials in ever growing quantities in consumer products and their presence in the environment, critical interest in the impact of this emerging technology has grown. The main concern is whether the unknown risks of engineered nanoparticles (NPs), in particular their impact on health and environment, outweighs their established benefits for society. Therefore, a key issue in this field is to evaluate their potential toxicity. In this context we evaluated the effects on plants and microorganisms of model nanoparticles, in particular of a stable metal (Au, 10 nm mean diameter), a well-known bactericide (Ag, 2 nm mean diameter) and the broadly used Fe3O4 (7 nm mean diameter). The toxicity of these nanoparticles was assayed using standard toxicity tests. Specifically, germination (cucumber and lettuce), bioluminescent (Photobacterium phosphoreum) and anaerobic toxicity tests were performed. Germination tests were conducted at a NP dose of 62, 100 and 116 µg mL\(^{-1}\) for Au, Ag, and Fe3O4, respectively. Finally, anaerobic tests were conducted at a NP dose of 10, 16 and 18 µg mL\(^{-1}\) for Au, Ag, and Fe3O4, respectively. In all cases low or zero toxicity was observed. However, some perturbation of the normal functions with respect to controls in germinating tests was observed, suggesting the necessity for further research in this field. At the same time, the effect of NP-solvents was sometimes more significant than that of the NPs themselves, a point that is of special interest for future nanotoxicological studies.

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1. Introduction

The use of nanoparticles (NPs) in commercial products and industrial applications has increased greatly in recent years although understanding of the interaction mechanisms at the molecular level between NPs and biological systems, is largely lacking (Maynard, 2006). In some of these products, such as skin creams and toothpastes, nanoparticles are in direct contact with the user’s body or can enter the environment on a continual basis from the removal (e.g. by washing) of such products (Daughton and Ternes, 1999) or, even worse, a fatal accident during the production of engineered nanomaterials could release an important quantity of nanoparticles to the environment (Moore, 2006).

At the same time, scientists have also found ways of using nanomaterials in environmental remediation. Although many of these are still in the testing phase (Ngomsik et al., 2005; Uheida et al., 2006; Li et al., 2006), dozens of sites have already been injected with various nanomaterials, including polymers or TiO₂, long used to mineralize many undesired organic pollutants (Mach, 2004). Recently, iron NPs were proposed as a low-cost technology for removing arsenic from drinking water (Yavuz et al., 2006). However, as noted by Lecoanet et al. (2004), nanosized materials may not migrate through soils at rapid enough rates to be valuable in remediation, and at the same time they may, in fact, become new environmental hazards. For example, TiO₂ absorbs substantial UV radiation yielding, in aqueous media, hydroxyl species. These species may cause substantial damage to DNA (Dunford et al., 1997; Hidaka et al., 1997), resulting in additional environmental hazards.

Most of the work performed on the toxicology of nanoparticles has been carried out using higher organisms, such as mice or fish as targets or only on the few species that have been accepted by regulatory agencies as models for defining ecotoxicologic effects.

Tests with uncoated, water-soluble, colloidal fullerenes (C60) show that the 48-h LC50 (median lethal concentration) in Daphnia magna is 800 ppb (Oberdorster, 2004a). In largemouth bass (Micropterus salmoides), although no mortality was seen, lipid peroxidation in the brain and glutathione depletion in the gill were observed after exposure to 0.3 µg mL\(^{-1}\) for 48 h (Oberdorster, 2004b). According to these results, toxic effects are highly dependant on the target organism, emphasizing that toxicity testing should be performed on a wide variety of organisms.
Other studies found that Al$_2$O$_3$ NPs reduced root growth due to the perturbation of the microbial composition of soil (Yang and Watts, 2005), raising concerns since the basis of many food chains depends on the benthic and soil flora and fauna, which could be affected by such NPs. Further, when Yang and Watts (2005) used root elongation tests on C. sativus to assess the toxicity of alumina nanoparticles, the results demonstrated that, under their experimental conditions, aluma nanoparticles do not induce any detectable effects on the seed root growth. Other authors (Lin and Xing, 2007) studied the effects of Zn and ZnO nanoparticles in seed germination and root elongation tests. Here the results indicated that Zn and ZnO nanoparticles caused significant inhibition of seed germination and root growth. In this area, Warheit et al. (2007) proposed a base set of toxicity tests to determine TiO$_2$ risk management.

Since available information on nanotoxicology is scarce, any scientific contribution on environmental risks of nanoparticles should help to regulate the use and production of nanoengineered materials. The objective of this work is thus to provide new data to evaluate the risks of the release to the environment of three representative metal-based nanoparticles, namely Fe$_3$O$_4$, Ag and Au nanoparticles. These nanoparticles are commonly used in commercial nanoengineered materials as antibactericide coatings, catalysts, in biomedicine or for personal care products. However, little data is found in the literature about their toxicity as evaluated by standard tests. To investigate environmental risks and implications, their phytotoxicity as measured by germination tests and toxicity in anaerobic and aerobic environments has been studied by means of several standard methods.

2. Experimental methods

2.1. Synthesis of nanoparticles

Three different kinds of inorganic NPs were synthesized in the aqueous phase, using milli-Q grade water. All reagents were purchased from Sigma-Aldrich and used as received. Briefly, for Au-NPs, injection of 1 mL gold tetrachloroaurate trihydrate (HAuCl$_4$·3H$_2$O) 23.4 mM into a 150 mL boiling solution containing 2.2 mM trisodium citrate yields monodisperse Au-NPs of 10 nm mean diameter. For Ag-NPs, injection of 2.64 mL of sodium borohydride (NaBH$_4$) 0.1 M in a solution of silver nitrate (AgNO$_3$) 0.1 mM chased from Sigma–Aldrich and used as received. Briefly, for Au-NPs, injection of 2.64 mL of sodium borohydride (NaBH$_4$) 0.1 M in a solution of silver nitrate (AgNO$_3$) 0.1 mM chased from Sigma–Aldrich and used as received, for Au-NPs, injection of 2.64 mL of sodium borohydride (NaBH$_4$) 0.1 M in a solution of silver nitrate (AgNO$_3$) 0.1 mM chased from Sigma–Aldrich and used as received. Briefly, for Au-NPs, injection of 2.64 mL of sodium borohydride (NaBH$_4$) 0.1 M in a solution of silver nitrate (AgNO$_3$) 0.1 mM chased from Sigma–Aldrich and used as received.

2.2. Stability of NPs: dynamic light scattering, Z-potential and microscopy

We determined the size distribution of suspended nanoparticles at various concentrations under different experimental conditions. The aim was to ascertain whether there is a time dependent agglomeration of nanoparticles after various incubation times and in different suspensions, since agglomerated NPs have different properties to those in monodisperse form. The NP suspensions were analyzed with dynamic light scattering (DLS) to determine NPs size distribution (and therefore agglomeration) in a Nanoparticle Analysis System (Malvern, UK). DLS is a well-known tool to determine the hydrodynamic diameter of colloidal particles. It is defined as the diameter of the sphere with the same Brownian motion as the analyzed particle. Zeta Potential (ZP) measurements were also performed for the same purpose. ZP measurements are a useful technique to study NPs stability and their surface charge in colloids when they are electrostatically stabilized. This technique operates like an electrophoresis. The Zetasizer applies an electric field across the sample and charged particles move towards the electrode of opposite charge with a characteristic velocity known as the electrophoretic mobility, which is converted into the Zeta Potential using Henry’s equation. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of the samples were also taken after NPs synthesis, to characterize the NPs, and after the toxicity experiments. In all cases the sizes of NPs responded similarly before and after the experiments.

2.3. Bioluminescent test

A Microtox® system from Microbics Corporation was used. This method is based on the percentage decrease in the amount of light emitted by the bioluminescent marine bacterium Photobacterium phosphoreum upon contact with a filtered sample at pH 7. Toxicity is, then, inversely proportional to the intensity of light emitted after the contact with the toxic substances (AFNOR T 90-320, AFNOR, 1991). The effective concentration, EC$_{50}$, is defined as the concentration that produces a 50% light reduction. EC$_{50}$ was measured after 5 and 15 min contact time. Results are expressed in equitox $\mu$mol-NP mL$^{-1}$ (100/EC$_{50}$). Toxicity tests for solvent samples and suspensions of nanoparticles (Table 2) were performed in triplicate. The pH values of solvents and nanoparticles suspensions were previously adjusted to 7. No visible precipitate was observed during the adjustment. Bioluminescent tests were performed at a sodium chloride concentration of 22% according to the manufacturer’s instructions. No visible precipitate was observed during the test, which confirmed the stability of the nanoparticles during the test period. Furthermore, TEM analysis after the experiment did not show any aggregation.

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Concentration (mM)</th>
<th>Size (mean diameter in nm)</th>
<th>Molar nanoparticle concentration (nM)</th>
<th>Mass concentration of element or molecule (μg mL$^{-1}$)</th>
<th>Solvent solution and concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>2.4 $10^{-12}$</td>
<td>10.1 ± 4.2</td>
<td>4</td>
<td>62</td>
<td>Trisodium citrate (2.2)</td>
</tr>
<tr>
<td>Ag</td>
<td>1.5 $10^{-12}$</td>
<td>28.2 ± 11.5</td>
<td>2</td>
<td>100</td>
<td>Sodium borohydride (2.64)</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>2.9 $10^{-13}$</td>
<td>7.57 ± 5.6</td>
<td>330</td>
<td>116</td>
<td>Tetramethylammonium hydroxide (10)</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of nanoparticles and their solvent.
2.4. Seed germination test

The phytotoxicity of NPs was evaluated by the seed germination technique. The germination index has been extensively used as an indicator of phytotoxicity in soils (Tiquia et al., 1996; Tiquia and Tam, 1998). Cucumber (Cucumis sativus) and lettuce (Lactuca sativa) seeds were used for this test. The seed germination percentage and root length of 10 cucumber seeds and 15 lettuce seeds were determined after 7 d of incubation at 25 °C.

The seed germination percentage and root elongation of both seeds in distilled water were also measured and used as a control. Experiments were done in triplicate. Final concentrations of NPs are shown in Table 2. The percentages of relative root elongation (E) and germination index (GI) were calculated according to standard methods using Eqs. (1)–(3) (Tiquia et al., 1996; US Department of Agriculture and US Composting Council, 2001)

Relative root elongation (E) = \[\frac{\text{Mean root length with NPs}}{\text{Mean root length with control}} \times 100\] (1)

Germination index (GI) = \[\frac{\text{Relative seed germination}}{100}\] (2)

where

Relative seed germination = \[\frac{\text{Seeds germinated with NPs}}{\text{Seeds germinated with control}} \times 100\] (3)

It is important to note that the germination index combines germination and root growth and consequently and therefore reflects the toxicity more completely. The root elongation is the percentage of root length compared to control and it can be an indication of the presence of stress effects or other non-acute toxicological effects in the plant evolution. Hence, the root elongation can be more sensitive than the germination index when the toxicity directly affects the root development.

2.5. Anaerobic toxicity test

The toxicity test in an anaerobic environment was performed by determining the percentage of decrease in the amount of biogas produced by a consortium of anaerobic bacteria. The test methodology to determine anaerobic toxicity was adapted from the Deutsche standard DIN 38414 (1987). The anaerobic inoculum was obtained from an industrial anaerobic digester treating the organic fraction of municipal solid wastes. Previous to its use, the inoculum was maintained during 15 d without feeding at 37 °C. Anaerobic assays were performed in 350 mL gas tight reactors, equipped with a pressure transducer to monitor biogas production (Ferrer et al., 2004). Each anaerobic reactor contained: 50 mL of inoculum, 50 mL of sample (solvent or nanoparticles suspension – see Table 2 for final concentrations), 1 g of cellulose and water to 300 mL. pH of each reactor was adjusted to 8 (if necessary) and nitrogen gas was used to purge oxygen previous to incubation to 37 °C during 21 d. Reactors were manually stirred and biogas was purged every workday. A blank and a reference test were also performed. The blank test (50 mL of inoculum and water to 300 mL) was performed to subtract biogas production from biodegradable organic matter contained in the inoculum. The control test (50 mL of inoculum, 1 g of microcrystalline cellulose and water to 300 mL) was performed to compare biogas production with sample tests. The use of microcrystalline cellulose as carbon source for anaerobic bacteria gives information about the possible effect of nanoparticles on the whole anaerobic bacterial consortium (hydrolytic, acidogenic, acetogenic and methanogenic bacteria) (Ahring, 2003).

2.6. Statistical data analysis

All toxicity tests were performed in triplicate. The statistical significance of values was checked by means of the Levene’s F-test (variance analysis) and Student’s t-test (mean analysis) both at 5% level of probability using the SPSS 15.0 package software (SPSS International, Chicago, IL). Statistically significant differences were reported when the probability of the result assuming the null hypothesis (p) is less than 0.05.

3. Results and discussion

In this work we selected different model NPs to test their effect on plants and microorganisms. The selected NPs were (i), Au, because of its known low reactivity and assumed biocompatibility. Au-NPs are being intensively developed as substrates for drugs in nanomedicine (Jain et al., 2007; Kogan et al., 2006), (ii) in contrast the Ag-NPs studied have been used as bactericide and microbiocide since ancient times. Ag-NPs attaches to the cell membrane of gram-negative bacteria creating lethal pores and producing bacteria lysis. Indeed, silver in its macroscopic form is already known to damage aquatic organisms (Braydich-Stolle et al., 2005). In the third class of NPs we measured the effects of (iii) Fe3O4-NPs since they have been recently proposed to be useful in advanced applications such as environmental remediation (Ngomsik et al., 2005; Uheida et al., 2006).

Table 2 shows NP concentrations used in each toxicity test. It has to be stated that toxicity effects of NPs at lower concentrations have been reported. For example, Ag-NPs at concentrations <0.1 μg mL⁻¹ are toxic to viruses, prokaryote and mammalian cells (Braydich-Stolle et al., 2005; Hussain et al., 2005).

3.1. NPs stability

For risk assessment, the dispersability and persistence of NPs are key parameters since they will determine how likely the living systems are to confront contaminants and hence determine their persistence and dispersion. Highly agglomerated NPs will travel less than monodisperse ones. In nanotechnology, there is a significant effort to obtain isolated NPs. This may be one of the critical
differences with the previously existing NPs, since natural and unintentional occurring NPs tend to agglomerate readily and because the physico-chemical differences between a granular material made of nanometric domains and an isolated NP are significant.

In toxicity tests it is important to control the stability of NPs since aggregation and/or sedimentation will modify the effective doses. In addition, the special physico-chemical properties that arise at the “nanolevel” (quantum confinement, superparamagnetism, extreme catalytic activity, etc.) are progressively/partially lost when NPs aggregate. Similarly, neither the properties nor the dynamics are similar. Agglomeration can lead to specific surfaces and concentrations very different from those of the original NP dispersion.

Stable NPs produced in the laboratory may become unstable when dispersed in different media. In the case of electrostatically stabilized NPs, which correspond to the non-functionalized inorganic NPs, presented in this work, the presence of salts in water or biological media may destabilize the particles. However, in all our studied cases, the stability of the NPs was not compromised at any stage of the experiments. DLS and TEM images show that, before and after the experiments, there are no relevant changes in the size distribution and stability of Au, Fe₃O₄ or Ag-NPs. Fig. 1a–c shows the main characteristics (diameter and Zeta Potential) of the used nanoparticles as they were synthesized. Further, the morphology, size distribution and shape of NPs did not show any significant change throughout the experiment. However, ZP measurements indicate that NPs have less negative charge when added to biological media (Fig. 1d and tables in Fig. 1). This modification in the ZP can be explained because the particle surfaces are coated with media molecules when the colloidal solution of NPs is added to the cell culture media (Thode et al., 1997). This modification of the surface, observed as a drop of the surface charge, means that NPs have molecules at their surface quenching the charge and simultaneously providing steric repulsion towards aggregation. This biomolecular coating is called a biomolecular or protein corona (Cedervall et al., 2007). In fact, a small increase of about 1 nm in particle size has been observed by DLS corresponding to this coating phenomenon. In the case of the seed growth experiments, NPs were absorbed onto the seed substrate. The red coloration in the case of Au-NPs indicated that no agglomeration occurred (Quinten and Kreibig, 1986; Storhoff et al., 2000). SEM observation of the substrates showed the NPs well distributed through the sample (not shown). In the case of the anaerobic assays, the solution was not transparent and optical measurements could not be performed. However, TEM observation after incubation indicated the absence of agglomeration. Thus, since no agglomeration of NPs was observed in any case, it can be concluded that the dosage of NPs did not change throughout the toxicity tests performed in this work.

3.2. Toxicity tests

The results obtained for each toxicity test are presented below. The effect of NPs on each organism (seeds, bioluminescent bacteria and anaerobic bacteria) was compared with a control test (NPs and

![Fig. 1. From left to right: TEM images, size distribution histograms and table with size distribution measured by TEM and DLS, and Zeta Potential values for: (a) Au-NPs (b) Ag-NPs (c) Fe-NPs (d) Au-NPs obtained from the anaerobic toxicity test medium (left) and Gold NPs as synthesized (right) and Zeta Potential values for Au-NPs (1) as synthesized and Au-NPs obtained from the anaerobic toxicity test medium. Scale bars correspond to 100 nm.](image-url)
solvent free) for each test. The effect of each neat NP-solvent (NPs free) was also studied.

### 3.2.1. Bioluminescent test

The first set of experiments was conducted to determine possible toxicity effects of nanoparticle suspensions and solvents in an aquatic environment. The bioluminescent test is widely used to evaluate the potential harmful effects of effluents discharged into surface waters (DIN 38412, 1991). Some proposed regulations set limits for bioluminescent toxicity at 25 Eqvitox m⁻³ (Generalitat de Catalunya, 2007).

Under the experimental conditions used neither solvents nor suspensions of nanoparticles presented toxic effects. In each case the toxic concentration was greater than the highest concentration assayed (45% of the initial concentration, see Table 2). This means that the toxicity of NPs samples was, in any case, <2.22 Eqvitox m⁻³.

### 3.2.2. Seed germination tests

Two different measurements were performed in this test (root elongation and germination index) using two different seeds (cucumber and lettuce). Table 3 summarizes the results obtained for the cucumber seeds germination test. As can be observed, except for Au-NPs and Au-solvent samples, all results suggest a significant ($p < 0.05$) reduction effect on germination index. In contrast, the Au-solvent sample produced a significantly ($p = 0.018$) positive influence on the germination index (96.7% vs. 116.4%). Results obtained for cucumber seeds root growth (Table 3) indicate that Ag-solvent, Ag-NPs and Fe-solvent produced a significant negative ($p = 0.016$ and $p = 0.012$, respectively) influence. Similarly to the germination index, the Au-solvent sample produced a significant ($p = 0.009$) positive effect on root growth. The other tested samples (Fe-NPs and Au-NPs) showed no significant differences compared to the control (distilled water) test.

Table 4 shows results obtained for lettuce seeds. Clearly, Ag-solvent, Fe-solvent and Fe-NPs produced a significant ($p < 0.05$) negative effect on the germination index. Au-NPs, in contrast, produced a significant ($p < 0.001$) positive effect on the germination index. In all cases solvent and nanoparticle pairs presented significant differences. All samples tested for root growth, except Ag-NPs, presented significant differences ($p < 0.05$) when compared with the reference test. Ag-solvent, Fe-solvent and Fe-NPs showed a negative effect, while Au-solvent and Au-NPs presented a positive effect.

Comparing all the results obtained in the seed germination test, it is evident that the toxic effect of NPs-solvent is more important than the NPs themselves. In addition, one can correlate the observed effects with the particular stabilizer present in the NP solutions: the poor biocompatibility of TMAOH or NaBH₄ and the biocompatible sodium citrate which is known to be a food additive (Table 2 shows stabilizer concentration present in each assayed toxicity tests). Therefore, the differences observed between the NPs solutions and the NP-free solutions (solvent) can be correlated to the adsorption of solvent molecules at the NPs surface decreasing the effective concentration of those molecules (TMAOH, sodium citrate, NaBH₄). Thus, the observed effect (positive or toxic) is, for the same concentration, less pronounced in the presence of NPs.

Fig. 2 shows the results obtained for the length root and weight root in the cucumber test. Although these parameters are not standardized in toxicity tests, they may be useful to compare the toxicity effects after seeds exposure to NPs since low values can be related to non-acute toxicological or stress effects. The pair Fe-NPs and Fe-solvent presented significantly higher values of root weight than elongation. On the contrary, in the pair Au-NPs and Au-solvent, root elongation values are higher than those of root weight. In consequence, it seems that in the case of Fe-NPs, the development of thicker roots was favored, whereas in the case of Au, root growth was mainly due to elongation. The root growth in length but not in width might be an avoidance mechanism of the seed to a stress factor produced by the presence of NPs. In any case, it is clear that length and weight root tests can be complementary for the description of plant toxicological stress. However, further research on the standardization of weight tests should be carried out to decide aspects such as germination time, number of seeds, minimum weight to be considered or use of total

![Author's personal copy](https://example.com/author-specific-image.png)

Table 3

<table>
<thead>
<tr>
<th>Cucumber</th>
<th>Germination index (%)</th>
<th>Significance (comparing sample with control)</th>
<th>Significance (comparing NPs with solvent)</th>
<th>Root elongation (%)</th>
<th>Significance (comparing sample with reference)</th>
<th>Significance (comparing NPs with solvent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (distilled water)</td>
<td>96.7 ± 21.5</td>
<td>$p = 0.016$</td>
<td>$p = 0.025$</td>
<td>100 ± 22.3</td>
<td>$p = 0.016$</td>
<td>$p = 0.978$</td>
</tr>
<tr>
<td>Ag-solvent</td>
<td>81.9 ± 22.2</td>
<td>$p = 0.001$</td>
<td>$p = 0.763$</td>
<td>84.7 ± 23.0</td>
<td>$p = 0.021$</td>
<td>$p = 0.624$</td>
</tr>
<tr>
<td>Ag-NPs</td>
<td>76.4 ± 17.8</td>
<td>$p = 0.001$</td>
<td>$p = 0.007$</td>
<td>84.9 ± 19.8</td>
<td>$p = 0.013$</td>
<td>$p = 0.272$</td>
</tr>
<tr>
<td>Fe-solvent</td>
<td>79.0 ± 23.1</td>
<td>$p = 0.001$</td>
<td>$p = 0.763$</td>
<td>84.6 ± 24.7</td>
<td>$p = 0.001$</td>
<td>$p = 0.624$</td>
</tr>
<tr>
<td>Fe-NPs</td>
<td>76.8 ± 27.4</td>
<td>$p = 0.001$</td>
<td>$p = 0.007$</td>
<td>88.6 ± 31.6</td>
<td>$p = 0.011$</td>
<td>$p = 0.272$</td>
</tr>
<tr>
<td>Au-solvent</td>
<td>116 ± 35.9</td>
<td>$p = 0.018$</td>
<td>$p = 0.272$</td>
<td>120 ± 37.2</td>
<td>$p = 0.001$</td>
<td>$p = 0.272$</td>
</tr>
<tr>
<td>Au-NPs</td>
<td>106 ± 32.0</td>
<td>$p = 0.016$</td>
<td>$p = 0.025$</td>
<td>110 ± 33.1</td>
<td>$p = 0.001$</td>
<td>$p = 0.272$</td>
</tr>
</tbody>
</table>

* Values correspond to average ± standard deviation obtained for all seeds from triplicates.

Table 4

<table>
<thead>
<tr>
<th>Lettuce</th>
<th>Germination index (%)</th>
<th>Significance (comparing sample with control)</th>
<th>Significance (comparing NPs with solvent)</th>
<th>Root elongation (%)</th>
<th>Significance (comparing sample with reference)</th>
<th>Significance (comparing NPs with solvent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (distilled water)</td>
<td>93.3 ± 32.7</td>
<td>$p = 0.007$</td>
<td>$p = 0.002$</td>
<td>100 ± 35.1</td>
<td>$p = 0.002$</td>
<td>$p = 0.002$</td>
</tr>
<tr>
<td>Ag-solvent</td>
<td>75.7 ± 26.9</td>
<td>$p = 0.001$</td>
<td>$p = 0.002$</td>
<td>78.3 ± 27.8</td>
<td>$p = 0.001$</td>
<td>$p = 0.002$</td>
</tr>
<tr>
<td>Ag-NPs</td>
<td>94.5 ± 29.0</td>
<td>$p = 0.001$</td>
<td>$p = 0.002$</td>
<td>97.7 ± 30.0</td>
<td>$p = 0.746$</td>
<td>$p = 0.001$</td>
</tr>
<tr>
<td>Fe-solvent</td>
<td>55.3 ± 13.1</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
<td>59.2 ± 14.0</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
</tr>
<tr>
<td>Fe-NPs</td>
<td>70.6 ± 18.2</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
<td>78.5 ± 20.2</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
</tr>
<tr>
<td>Au-solvent</td>
<td>105 ± 20.0</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
<td>118 ± 22.3</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
</tr>
<tr>
<td>Au-NPs</td>
<td>121 ± 27.0</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
<td>121 ± 27.0</td>
<td>$p = 0.001$</td>
<td>$p = 0.001$</td>
</tr>
</tbody>
</table>

* Values correspond to average ± standard deviation obtained for all seeds from triplicates.
or dry matter, since the experimental difficulties in determining root weights are important.

This seems to indicate that the toxicity effect of NP-solvent cannot be described using yes/no germination tests, and that a much more specific analysis of germination results is necessary to discover stress phenomena related to the presence of some apparently non-toxic compounds. For instance, Fig. 2 also shows that NPs seem to decrease the stress effect observed for NPs-solvent. Also, in the case of Ag, no statistical differences are observed among Ag-solvent and Ag-NPs and control (distilled water) values. Although more research should be carried out on the toxicity and stress effects after a long-time exposure to NPs, this is, to our knowledge, the first work in which these parameters have been proposed to analyze specific stress phenomena due to the presence of NPs.

3.2.3. Anaerobic toxicity test

Table 5 summarizes the results obtained with the proposed anaerobic toxicity test. The results presented correspond to the cumulative biogas production obtained after 21 d, (after subtracting biogas produced in the blank test). Control tests (inoculum with microcrystalline cellulose) produced 471 ± 19 l biogas kg DM⁻¹. According to the German standard DIN 38414, biogas production of the control test should be, at least, 400 l biogas kg DM⁻¹ to validate the activity of the anaerobic inoculum used. Additionally, the deviation values are below 20%, therefore the results obtained are considered validated.

Statistical analysis suggests that, at the assayed concentrations, Fe₃O₄, Ag, and Au nanoparticles do not have a significant effect on the anaerobic bacterial consortium, since, in any case, the biogas production was not significantly different (p < 0.05) from the control test. However, it was noted that the solvent used with Fe₃O₄ nanoparticles (Fe-solvent), produced significantly (p = 0.001) more biogas than the reference, indicating a positive effect on the anaerobic bacterial consortium. Also, in the case of Fe₃O₄ nanoparticles, significant differences could be detected between Fe-solvent and Fe-NPs, with a slightly less increased effect on biogas production in the case of Fe-NPs (557.1 vs 518.6 l biogas kg DM⁻¹). An additional biogas test with decreasing concentrations of Fe-NPs (from the value shown in Table 2) revealed no significant differences compared to the control test (data not shown). Values of biogas production were significantly different between Au-solvent and the control test, although biogas production for the Au-NPs was identical than those of control and Au-solvent.

Although no references to inorganic NPs in anaerobic environments have been found in the literature, recent studies have reported an absence of toxicity in anaerobic microorganisms using C₆₀ fullerenes (Nyberg et al., 2008). In this interesting study, it is
concluded that solvents used to stabilize nanomaterials can, in the same way as nanomaterials, play an important role in the slight disturbances observed in the anaerobic consortium.

3.3. General discussion

In this work, we have observed that toxicity effects can be due to the presence of NPs-solvent (stabilizers) and to the combined effect of NPs-solvent and NPs. While no effect of NPs in the bioluminescent test was observed, some effects were observed in the case of anaerobic bacteria (mainly in the case of NPs-solvents) and a modified root growth in the germination tests.

In the germination tests, in some cases a slight positive effect of NPs was observed, which can be due to a generally-favorable biological response to low exposures of toxins and other stressors (hormesis effect). Moreover, while the germination index was similar regardless of the NPs, their presence induced growth of larger roots. This might indicate that the seeds were slightly stressed by the environment, possibly resulting in harmful effects on long-term exposition.

Also, the importance, when performing toxicity tests, of maintaining the stability of NPs in the solvent medium, has been highlighted in our study. NPs stability must be assured by the addition of stabilizers. This being the case, one has to understand that engineered NPs in solution are always accompanied by stabilizers; otherwise their duration in solution is very short (Hyung et al., 2007). Our results indicate that the solvent effect diminishes when NPs are present. This might be explained by adsorption of NPs onto the surface of stabilizer molecules, thus reducing the concentration (dose) of available stabilizer.

Compared with literature data, while photoactive ZnO or TiO₂ (Warheit et al., 2007), bactericidal Ag (Shrivastava et al., 2007), hydrophobic Carbon nanotubes (Smith et al., 2007) and fullerences (Oberdorster, 2004a), or Cadmium oxide particles (Bradych-Stolle et al., 2005), show environmental toxicity, it appears that Au and iron oxide NPs are significantly less toxic.

In conclusion low or zero toxicity was observed for Au, Ag and Fe₃O₄ nanoparticles at the assayed concentrations. However, since NPs must be accompanied by stabilizers, in some cases a positive or negative effect was observed due to the presence of the latter (in our case TMAOH, sodium citrate, NaBH₄). Development of new nanoparticle surface engineering strategies should lead to more environmentally friendly nanoparticles. At the same time, the Copus et al. emphasize the need for a deeper understanding of the interaction of nanoparticulate inorganic materials with the environment prior to their massive industrial use.

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References


