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EVALUATION OF MODEL NANOPARTICLES ECO-TOXICITY

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ABSTRACT

Since society realized about the use of nanomaterials in greater quantities in consumer products and their presence in the environment, the interest on the impact of this emerging technology has grown. The main concern is whether the unknown risks of engineered nanoparticles (NPs), in particular their health and environmental impact, outweighs their established benefits for society. Therefore, a key issue in this field is to evaluate the potential toxicity of these engineered nanomaterials. 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 Fe_3O_4 (7 nm mean diameter). Toxicity of these nanoparticles was assayed by using standard toxicity tests. Specifically germination test (cucumber and lettuce), bioluminescent test (*Photobacterium phosphoreum*) and anaerobic toxicity tests has been performed. Germination tests were conducted at a NP dose of 62, 100 and 116 µg ml^{-1} for Au, Ag, and Fe_3O_4 respectively. Bioluminscent test (*Photobacterium phosphoreum*) was conducted at a dose of 28, 45 and 52 µg ml^{-1} for Au, Ag, and Fe_3O_4 respectively. Finally anaerobic tests were conducted at a NP dose of 10, 16 and 18 µg ml^{-1} for Au, Ag, and Fe_3O_4 respectively. In all cases low or nil toxicity was observed. However some perturbation of the normal functions with respect to controls in germinating tests was observed, suggesting the necessity of further research in this field. At the same time, the effect of NPs solvents was in some cases more significant than that of NPs themselves, a point that is of special interest for future nanotoxicology studies.

KEYWORDS anaerobic consortium; nanoparticles; *Photobacterium phosphoreum*; phytotoxicity; toxicity effects.

1. Introduction

The use of nanoparticles (NPs) in commercial products and industrial applications has increased in the last years without a full understanding of the interaction mechanisms at the molecular level between NPs and biological systems (Maynard, 2006). In some of these products, such as skin creams and toothpastes, nanoparticles are in direct contact with a person's body or can enter the environment on a continual basis from washing off of consumer 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). In those cases, what has been unclear, or ignored, so far, is that foreign bodies below a certain size can enter animal organisms, mainly through ingestion or respiration, they may either negotiate the gastro-intestinal wall, the skin or the pulmonary alveoli, be carried by the blood or the lymph and travel rather freely through tissues, or settle in a tissue they run into on their migratory way, and enter the food chain.

At the same time, scientists have also found ways of using nanomaterials in environmental remediation. Although many of these are still in testing stages (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 TiO_2, used for long to mineralize many undesired organic pollutants (Mach, 2004). Recently, iron NPs were proposed as low-cost technology for cleaning arsenic from drinking water, which holds promise for millions of people in India, Bangladesh and other developing countries where thousands of cases of arsenic poisoning each year are linked to poisoned wells (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, at the same time that they may represent a new environmental hazard. For example, TiO_2 absorbs substantial UV radiation which, in aqueous media, yields

hydroxyl species. These species may cause substantial damage to DNA (Dunford et al., 1997; Hidaka et al., 1997), what could result in environmental hazards.

Regarding the environmental models, most of the work performed on nanoparticles toxicology has been made using superior organisms as target, such as mice or fish 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-hour 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 0.5 ppm for 48 hours (Oberdorster, 2004b). Other studies found that Al_2O_3 NPs reduced root growth due to the perturbation of the microbicidal composition of soil (Yang and Watts, 2005), rising concerns since the basis of many food chains depends on the benthic and soil flora and fauna, which could be affected by such NPs. Besides, when Yang and Watts (2005) used root elongation tests to assess toxicity of alumina nanoparticles, results demonstrated that, under those experimental conditions, alumina 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 by seed germination and root elongation tests. Obtained results pointed that Zn and ZnO nanoparticles presented significant inhibition on 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 will help the regulation on the use and production of nanoengineered materials. The objective of this work is to provide new data to evaluate the risks of the release to the environment of three metal-based nanoparticles, such as Fe_3O_4, Ag and Au nanoparticles. These types of nanoparticles are commonly used in commercial nanoengineered materials. However, very few data is found in literature about their toxicity

evaluated by standard tests. To investigate environmental risks implications, phytotoxicity measured by germination tests and toxicity of nanoparticles in anaerobic and aerobic environments has been here studied by means of several standard methods.

2. Experimental Methods

2.1 Synthesis of nanoparticles

Three different kinds of inorganic NPs were synthesized in 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 tetrachloroaureate trihydrate ($HAuCl_4 \cdot 3H_2O$) 23.4 mM in a boiling solution containing 2.2 mM trisodium citrate yields to 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 ($AgNO_3$) 0.1mM in deoxygenated water yields to Ag-NPs of about 30 nm mean diameter. And for Fe_3O_4, amounts of 1 mmol iron (II) chloride ($FeCl_2$) and 2 mmol iron (III) chloride ($FeCl_3$) were dissolved in 10 ml deoxygenated water and then added dropwise to 10 ml of a solution of 1 M deoxygenated tetramethylammonium hydroxide (TMAOH). After 30 minutes of vigorously stirring under N_2 bubbling, the Fe_3O_4 precipitate was washed by soft magnetic decantation and redissolved in 1 M TMAOH to obtain the final stable colloidal solution of Fe_3O_4-NPs of 7 nm mean diameter. Characteristics of NPs and solvents are shown in Table 1. Table 2 shows the resulting concentrations exposed in the toxicity tests assayed in this work.

2.2 Stability of NPs: Dynamic Light Scattering, Z-potential and Microscopy

We determined the size distribution of suspended nanoparticles at various concentrations through the experiment conditions. The aim was to assay if there is a time dependent agglomeration of nanoparticles after various incubation times and in different suspensions,

since agglomerated NPs have different behavior than monodisperse ones. The NPs 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, which is the diameter of the sphere with the same Brownian motion as the analyzed particle. Zeta Potential (ZP) measurements were also performed for the same objectives. ZP measurements are a useful technique to study NPs stability and their surface charge in colloids when they are electrostaticly stabilized. This technique operates like an electrophoresis. The Zetasizer apply an electric field across the sample and charged particles moves towards the electrode of opposite charge with a characteristic velocity, the electrophoretic mobility, which is converted into the Zeta Potential using Henry's equation. Also, Transmission Electron Microscope (TEM) and Scanning Electron Microscopy (SEM) images of the samples were 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 of 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 m^{-3} ($100/EC_{50}$). Toxicity tests for solvent samples and nanoparticles suspensions samples (Table 2) were performed in triplicate. pH of solvents and nanoparticles suspension samples was previously adjusted to 7. No visible precipitate was observed during the adjustment. Bioluminescent tests were performed under a

sodium chloride concentration of 22% according to the manufacturer's instructions. No visible precipitate was observed during the test, which confirmed nanoparticles stability during the test period.

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. After 7 days of incubation of 10 seeds of cucumber and 15 seed of lettuce at 25ºC, the seed germination percentage and root length of the seeds were determined. The seed germination percentage and root elongation of both seeds in distilled water were also measured and used as a control experiment. 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 Equations 1-3 (Tiquia et al., 1996; U.S. Department of Agriculture and U.S. Composting Council, 2001):

Relative root elongation (E) = (Mean root length with NPs)/(Mean root length with control)x100 (1)

Germination Index (GI) = (Relative seed germination)x(Relative root elongation)/100 (2)

where:

Relative seed germination = (Seeds germinated with NPs)/(Seeds germinated with control)x100 (3)

It is important to note that the germination index combines germination and root growth and consequently it is a more complete toxicity parameter. 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 germination index when the toxicity affects directly the root development.

2.5 Anaerobic toxicity test

The toxicity test in 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 Deustch standard DIN-38414 (1987). The anaerobic inoculum was obtained from an industrial anaerobic digester treating organic fraction of municipal solid wastes. Previously to its use, inoculum was maintained during 15 days without feeding at 37ºC. 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 37ºC during 21 days. 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 food for anaerobic bacteria gives information of 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. Statistical significance of values was checked by means of the Levene F-test (variance analysis) and t-Student 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

In this work we selected different model NPs to test their effect on plants and microorganisms. The selected NPs were firstly, Au because of its known low reactivity and assumed biocompatibility, which is intensively being developed as substrate for drugs in nanomedicine (Jain et al., 2007; Kogan et al., 2006). On the opposite side there are Ag-NPs used as bactericide and microbicide 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 addition we measure the effects of Fe_3O_4-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 NPs concentration used in each toxicity test. Although concentrations may seem small, it has been reported toxicity effects of NPs at lower concentrations have been reported. For example, Ag-NPs at concentrations below 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 is a key parameter since it will determine how likely are the living systems susceptible to confront contaminants and it will

determine the persistence and spread of the NPs. Highly agglomerating 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 NPs stability 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 leads to specific surfaces and concentrations very different from the parent NPs dispersion.

Stable NPs produced in the laboratory may become unstable when dispersed in different media. In the case of electrostatic stabilized NPs, what corresponds to non-functionalized inorganic NPs, as the ones presented in this work, the presence of salts in water or biological media may destabilize the particles. However, in all the 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, Fe_3O_4 or Ag NPs. Figure 1a and 1b presents Au-NPs before and after the anaerobic toxicity test. It can be observed that 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 (Figure 1c). This modification in the ZP can be explained because the particle surfaces are coated with media molecules when the NPs colloidal solution 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 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 seeds substrate. The red coloration in the case of Au-NPs indicated that no agglomeration occurred. 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 NPs dose did not change along the toxicity tests performed in this work.

3.2 Toxicity tests

Results obtained for each toxicity test are explained below. The effect of NPs on each organism (seeds, bioluminescent bacteria and anaerobic bacteria) was compared with a control test (NPs and solvent free) for each test. The effect of 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 nanoparticles suspension and solvents in an aquatic environment. The bioluminescent test is broadly used to evaluate the potential harmful effects of effluents discharged into surface waters (DIN-38412, 1991). Some proposed regulations set limit values for bioluminescent toxicity at 25 Equitox m^{-3} (Generalitat de Catalunya, 2007).

Under the experimental conditions used neither solvents nor nanoparticles suspension presented toxic effects. In all cases toxicity concentration was greater than the highest

concentration assayed (45% of the initial concentration, see Table 2). That means that, toxicity of NPs samples was, in any case, lower than 2.22 Equitox m^{-3}.

3.2.2 Seed germination tests

Two different measures 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. On the contrary 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 negative significant ($p=0.016$ and $p=0.012$ respectively) influence. Similarly to germination index, Au-solvent sample produced a significant ($p=0.009$) positive effect on root growth. The other tested samples (Fe-NPs and Au-NPs) did not present significant differences compared to the control (distilled water) test.

Table 4 shows results obtained for lettuce seeds. As can be observed, Ag-solvent, Fe-solvent and Fe-NPs produced a significant ($p<0.05$) negative effect on germination index. On the contrary Au-NPs produced a significant ($p<0.001$) positive effect on germination index. In all cases solvent and nanoparticles pairs presented significant differences. All samples tested for root growth, except Ag-NPs, presented significant differences ($p<0.05$) when were 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 toxicity 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 NPs solutions: the poor biocompatibility of TMAOH or $NaBH_4$ 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, $NaBH_4$). Thus, the observed effect (positive or toxic) is, for the same concentration, less pronounced in the presence of NPs.

Figure 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 can be stated 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. Anyway, it is clear that length and weight root tests can be complementary for the description of plant toxicological stress. However, a research work 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 or dry matter, since the experimental difficulties in determining root weights are important.

This seems to indicate that the toxicity effect of NPs-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, Figure 2 also shows that NPs seems 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 where 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. Results presented correspond to the cumulative biogas production obtained after 21 days, subtracting biogas produced in the blank test. Control tests (inoculum with microcrystalline cellulose) produced 471 l biogas kg DM^{-1}. According to the Deustch 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 validated.

Statistical analysis suggests that, at the assayed concentrations, Fe_3O_4, Ag and Au nanoparticles do not have a significant effect on anaerobic bacterial consortium, since biogas production was not in any case significantly different ($p<0.05$) from the control test. However, it was detected that the solvent used with Fe_3O_4 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 Fe_3O_4 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 of inorganic NPs in anaerobic environments have been found in literature, recent studies have observed an absence of toxicity in anaerobic microorganisms using C_{60} fullerenes (Nyberg et al., 2008). In this interesting study, it is concluded that solvents used to stabilize nanomaterials can play an important role in some slight disturbances observed in the anaerobic consortium equal to nanomaterials.

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 observed effect of NPs in the bioluminescent test, 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. Observed effects were either positive or negative.

In the germination tests, in some cases a slight positive effect of NPs was observed, which can be due to a hormesis effect, that is, a generally-favorable biological responses to low exposures to toxins and other stressors. Moreover, while the germination index was similar regardless of the NPs, the presence of them induced growth of larger roots as if the seeds were slightly stressed by the environment, which in the long term may be harmful, depending on the persistence of NPs in the environment.

Also, the importance of ensuring the stability of NPs in the media, when performing toxicity tests, has been highlighted. NPs stability must be assured by the addition of stabilizers. Then, one has to understand that engineered NPs in solution are always accompanied by stabilizers; otherwise their permanence in solution is very short (Hyung et al., 2007). Presented results indicate that solvent effect is faded when NPs are present. This could be explained due to NPs adsorption of stabilizers molecules on his surface, then reducing the concentration (dose) of available stabilizer.

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

In conclusion low or nil toxicity effect was observed for Au, Ag and Fe_3O_4 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 these molecules (TMAOH, sodium citrate, $NaBH_4$). Presented results indicate the need to a deeper understanding of the interaction of nanoparticulate inorganic material with the environment prior to massive industrial use of nanomaterials.

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Figure Captions

Figure 1: Z-potential Graphs before and after the experiments and Transmission Electron Microscope (TEM) of Au NPs samples in the anaerobic toxicity test: a) TEM image of synthesized Au NPs ; b) TEM image of Au NPs after anaerobic toxicity test (bars are 200 nm); c) ZP graphs of synthesized Au NPs (peak 1 = -41.0 mV) and after the experiment (peak 2 = -20.1 mV).

Figure 2: Comparison of germination index and root elongation with respect to root length and root weight. Error bars correspond to standard deviation found for root length values.

Figure 1.-

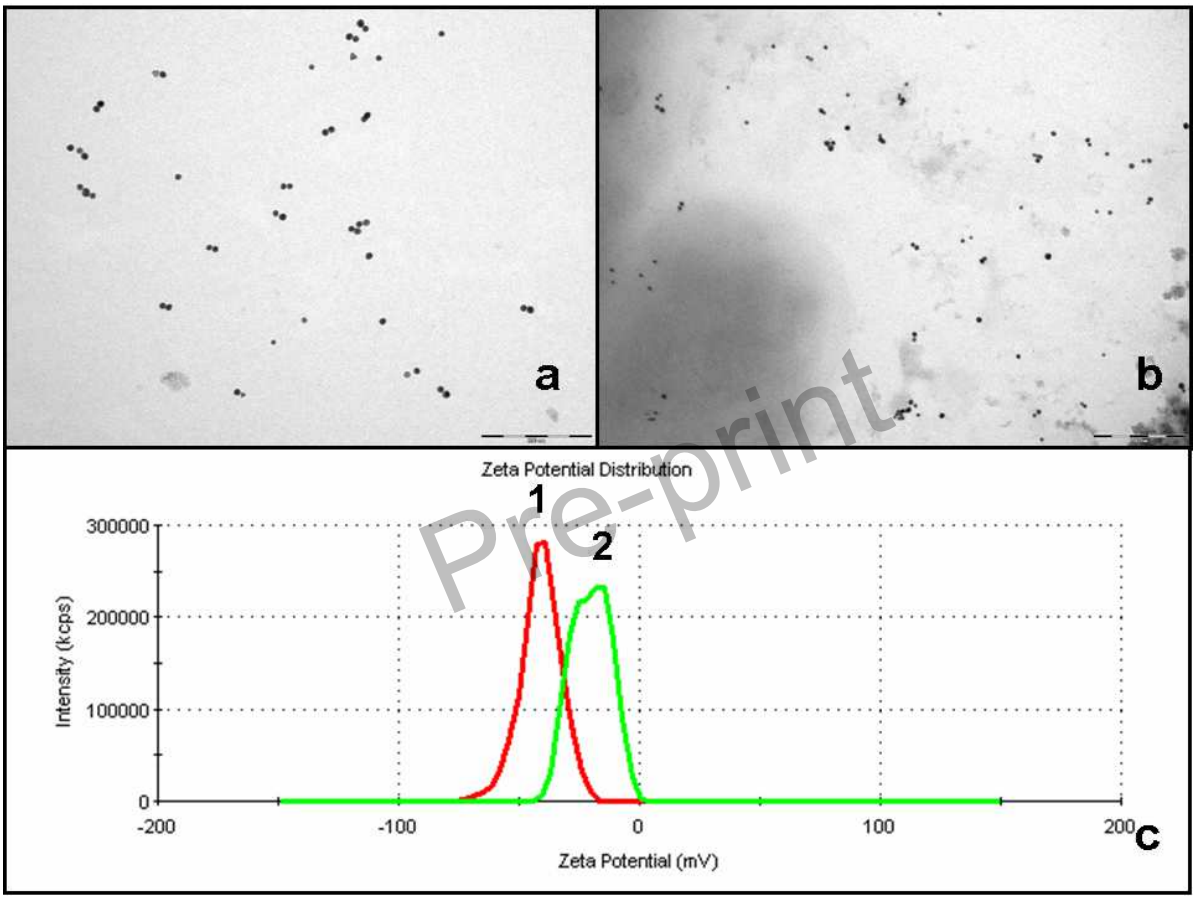
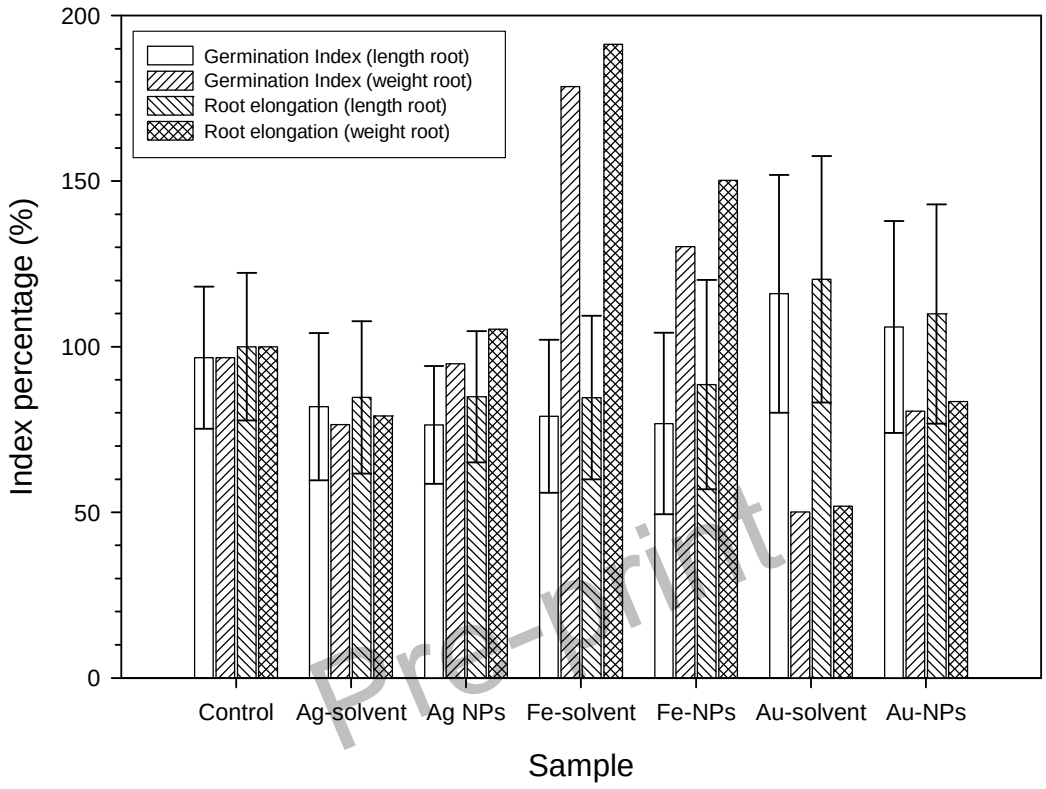


Figure 2.-



Tables

Table 1. Characteristics of nanoparticles and nanoparticles solvent.

Nanoparticle	Size (mean diameter in nm)	Molar nanoparticle concentration (nM)	Mass concentration of element or molecule ($\mu g\ ml^{-1}$)	Solvent solution and concentration (mM)
Au	10	4	62	Trisodium citrate (2.2)
Ag	30	2	100	Sodium borohydride (2.64)
Fe_3O_4	7	330	116	Tetramethylammonium hydroxide (10)

Table 2. Concentrations of nanoparticles used in the toxicity assays.

Sample	Bioluminescent test (45% of the initial concentration)	Germination test (100% of the initial concentration)	Anaerobic test (16% of the initial concentration)
Au (µg ml^{-1})	28	62	10
Ag (µg ml^{-1})	45	100	16
Fe_3O_4 (µg ml^{-1})	52	116	18
Au-stabilizer: trisodium citrate (mM)	0.99	2.2	0.35
Ag-stabilizer: sodium borohydride (mM)	1.18	2.64	0.42
Fe_3O_4-stabilizer: tetramethylammonium hydroxide (mM)	4.5	10	1.6

Table 3. Influence of nanoparticles samples on germination index and root growth for cucumber seeds.

Cucumber	Germination index[¥] (%)	Significance (comparing sample with control)	Significance (comparing NPs with solvent)	Root elongation[¥] (%)	Significance (comparing sample with reference)	Significance (comparing NPs with solvent)
Control (distilled water)	96.7 ± 21.5			100 ± 22.3		
Ag-solvent	81.9 ± 22.2	p=0.016	p=0.325	84.7 ± 23.0	p=0.016	p=0.978
Ag-NPs	76.4 ± 17.8	p<0.001		84.9 ± 19.8	p=0.012	
Fe-solvent	79.0 ± 23.1	p=0.006	p=0.763	84.6 ± 24.7	p=0.021	p=0.624
Fe-NPs	76.8 ± 27.4	p=0.007		88.6 ± 31.6	p=0.139	
Au-solvent	116 ± 35.9	p=0.018	p=0.272	120 ± 37.2	p=0.018	p=0.272
Au-NPs	106 ± 32.0	p=0.196		110 ± 33.1	p=0.196	

[¥] Values correspond to average ± standard deviation obtained for all seeds from triplicates.

Table 4. Influence of nanoparticles samples on germination index and root growth for lettuce seeds.

Lettuce	Germination index[¥] (%)	Significance (comparing sample with control)	Significance (comparing NPs with solvent)	Root elongation[¥] (%)	Significance (comparing sample with reference)	Significance (comparing NPs with solvent)
Control (distilled water)	93.3 ± 32.7			100 ± 35.1		
Ag-solvent	75.7 ± 26.9	p=0.007	p=0.002	78.3 ± 27.8	p=0.002	p=0.002
Ag-NPs	94.5 ± 29.0	p=0.846		97.7 ± 30.0	p=0.746	
Fe-solvent	55.3 ± 13.1	p<0.001	p<0.001	59.2 ± 14.0	p<0.001	p<0.001
Fe-NPs	70.6 ± 18.2	p<0.001		78.5 ± 20.2	p=0.001	
Au-solvent	105 ± 20.0	p=0.036	p=0.004	118 ± 22.3	p=0.007	p=0.532
Au-NPs	121 ± 27.0	p<0.001		121 ± 27.0	p=0.002	

[¥] Values correspond to average ± standard deviation obtained for all seeds from triplicates.

Table 5. Biogas production and statistical analysis for the anaerobic toxicity test.

Sample	Biogas production (l kg $DM^{-1(*)}$)(¥)	Significance (comparing sample with control)	Significance (comparing NPs with solvent)
Control (microcrystalline cellulose)	471 ± 19	-	
Ag-solvent	482 ± 32	p=0.646	p=0.440
Ag-NPs	474 ± 44	p=0.607	
Fe-solvent	557 ± 84	p=0.002	p=0.010
Fe-NPs	519 ± 22	p=0.048	
Au-solvent	423 ± 14	p=0.041	p=0.098
Au-NPs	509 ± 62	p=0.406	

(*) DM: dry matter

(¥) Values correspond to average ± standard deviation obtained for all tests from triplicates.