Effect of cerium dioxide, titanium dioxide, silver, and gold nanoparticles on the activity of microbial communities intended in wastewater treatment

Ana García[a], Lucia Delgado[a], Josep A. Tora[a], Eudald Casals[b], Edgar González[b], Víctor Puntes[b,c], Xavier Font[a], Julián Carrera[a], Antoni Sánchez[a,*]

[a] Department of Chemical Engineering, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[b] Institut Català de Nanotecnologia, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[c] Institut Català de Recerca i Estudis Avançats, Passeig Lluís Companys, 23, 08010 Barcelona, Spain

*Corresponding author:

Email: antoni.sanchez@uab.cat

Telephone: +34 93 581 1018 Fax: +34 93 581 2013

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Abstract

Growth in production and use of nanoparticles (NPs) will result increased concentrations of these in industrial and urban wastewaters and, consequently, in wastewater-treatment facilities. The effect of this increase on the performance of the wastewater-treatment process has not been studied systematically and including all the microbial communities involved in wastewater treatment. The present work investigates, by using respiration tests and biogas-production analysis, the inhibitory effect of four different commonly used metal oxide (CeO_2 and TiO_2) and zero-valent metal (Ag and Au) nanoparticles on the activity of the most important microbial communities present in a modern wastewater-treatment plant. Specifically, the actions of ordinary heterotrophic organisms, ammonia oxidizing bacteria, and thermophilic and mesophilic anaerobic bacteria were tested in the presence and absence of the nanoparticles. In general, CeO_2 nanoparticles caused the greatest inhibition in biogas production (nearly 100%) and a strong inhibitory action of other biomasses; Ag nanoparticles caused an intermediate inhibition in biogas production (within 33-50%) and a slight inhibition in the action of other biomasses, and Au and TiO_2 nanoparticles caused only slight or no inhibition for all tested biomasses.

Keywords: Inorganic nanoparticles; inhibition; respirometry; anaerobic biomass; ammonia oxidizing bacteria; ordinary heterotrophic organisms.

1. Introduction

The use of nanoparticles (NPs) in many industrial applications including commercial products and water treatment has continuously increased in recent years [1,2]. This increased usage means that an increasing number of nanoparticles will be released to the environment through production processes or after their use [3,4]. Uncertainty about the consequences of the presence of the nanoparticles on the environment has initiated studies on the effects of nanomaterials by some facilities that use microorganisms for environmental restoration [5,6] and, in general, on the flow of nanoparticles through production processes and their various applications [7]. Several studies have been reported which aimed to determine the toxicity of nanoparticles on different sentinel organisms such as *Daphnia magna* [8], bioluminescent bacteria, and different plant seeds [9-12].

There have been some studies on the entire lifecycle of nanoparticles including production, use, and release into the environment, for example, for silver and zinc oxide [13,14]. In these studies it was shown that a portion of the released nanoparticles finally ends up in the wastewater-collection systems and then enters biological wastewater-treatment plants (WWTP) [15,16]. Currently, the concentration of silver in WWTP has been calculated to be in the range of 2 to 18 µg L^{-1} [13]. Titanium nanomaterial concentration has been measured in WWTP influent at 185 µg L^{-1} [17]. Different treatment operations (bar screen, grit removal, primary settling, etc.) could help to remove nanoparticles from wastewater [4], however, it has been demonstrated that nanoparticles can also be found in sewage sludge [18].

Little work has been carried out on the effect of nanoparticles on the different microbial populations that can exist in a biological WWTP. Cerium oxide nanoparticles

and heterotrophic bacteria [19] or silver nanoparticles and nitrifying bacteria [20-24] are examples of the model nanoparticles and the model biomass assayed. Both respirometric assays and scanning transmission electron microscopy have been used to demonstrate the interaction between nanoparticles and such microorganisms [19,20]. Information gleaned in this way should help in the regulation of production and use of nanoparticles as well as to estimate the potential risk on environment. Only through such rigorous studies can the rational development of nanotechnology be implemented [4,5].

The aim of this work is to provide new data to evaluate if there is an inhibitory effect in the use of two different metal oxide (CeO_2 and rutile TiO_2) and two zero-valent metal (Ag and Au) nanoparticles on the activity of the most important microbial communities involved in a WWTP. The overall effect on the facility where these microbial communities are used is also discussed. The choice of materials and the methods for their preparation and synthesis was made to model even the most complex materials used at present. We adjusted the final characteristics of the synthesized NPs (size between 10 and 30 nm, and similar in shape) to be comparable as far as possible with each other. With the aim of observing the maximum toxicological effect that nanoparticles can produce in the biological activity of the microbial communities studied and of calculating the EC_{50} value, higher concentrations than those generally found in a WWTP were also tested. Knowledge of the EC_{50} value for each NP should enable us to anticipate changes in the performance of practical wastewater treatment processes when such concentrations may arise; after accidental spill, for example.

2. Materials and methods

2.1 Preparation of nanoparticles

Four different kinds of metal oxide and zero-valent metal nanoparticles were synthesized in the aqueous phase, using milli-Q grade water. All reagents were purchased from Sigma-Aldrich and used as received. All the synthesis procedures were based on existing ones available in the scientific literature, with modifications to adapt to the large-scale (from milligram to gram).

For cerium oxide nanoparticles (CeO_2-NPs), the procedure was based on ref. [25]. Ce^{3+} ions from $Ce(NO_3)_3$ were oxidized under alkaline pH conditions to Ce^{4+} using hexamethylenetetramine (HMT). CeO_2 nanocrystals precipitated and were stabilized in water with the same reagent (HMT), which forms a double electrical layer to prevent nanoparticle aggregation.

For titanium dioxide nanoparticles (TiO_2-NPs), the synthesis procedure was based on ref. [26]. Titanium tetrachloride ($TiCl_4$) was decomposed at acidic pH (from 2 to 6). Afterwards, the growth of the nanocrystals was carried out in an oven at 70ºC. Finally, a purification step involving centrifugation and re-suspension with tetramethylammonium hydroxide (TMAOH) was used to stabilize the nanoparticle dispersion. Depending on the pH during the growing step, the obtained size and shape of the TiO_2 varied from very small and sphere-like (from 5 nm, not used in this work) to larger particles (around 10 nm, used in this work).

The 10-nm gold nanoparticles (Au-NPs) were obtained by using a procedure based on ref. [27], which consisted of the fast injection of 1 mL of a 0.01% hydrogen tetrachloroaureate (III) ($HAuCl_4$) solution to a boiling solution containing 100 mL of 0.8 % trisodium citrate ($Na_3C_6H_5O_7$) under vigorous stirring.

The same method was used to obtain the silver nanoparticles (Ag-NPs): injection of trisodium citrate to a solution of 1 mM silver nitrate ($AgNO_3$) in

deoxygenated water resulting in a final concentration of 10 mM yielded Ag-NPs of about 30 nm average diameter.

The characteristics of the nanoparticles and the stabilizers used in this work are shown in Table 1. The pH values of all the nanoparticles were typically slightly alkaline and these were adjusted to 7.5 using citric acid (1 M) before the toxicity experiments, to emulate conditions in the WWTP. Some strong acids were initially used for adjustment of pH but these rapidly caused nanoparticle agglomeration, probably because of the rapid formation of acidic zones prior to total acid dilution. It seems that citric acid, which is a very weak acid, does not alter the structure of nanoparticles and thereby prevents their agglomeration and precipitation, which can be further avoided for more than one month by using a suitable stabilizer [28]. The amount of sodium citrate required to sufficiently adjust the pH value was very small (few milligrams), which means that the contribution to the biochemical oxygen demand (BOD) derived from this compound was negligible. Nanoparticles have a high surface energy and the use of HMT, sodium citrate, and TMAOH as nanoparticle stabilizers is needed to provide sufficient electrostatic charge on the surface of the nanoparticles to avoid aggregation. At the concentrations used, the stabilizers were found to be non-toxic towards human cell lines [29] and other microbial organisms similar to those tested in this study [10] although, to our knowledge, their toxicity on the specific microbial communities of WWTP still has not been tested. The specific effect of these stabilizers on the activity of all microbial populations considered in this study, which are necessary to maintain the nanomaterial structure during the toxicity tests, were separately determined by means of control experiments for each microbial community tested.

2.2. Characterization and stability of nanoparticles

NPs were analyzed by using dynamic light scattering (DLS) to determine their size distribution and whether agglomeration had occurred at any moment during the experimental runs (more than 1 month). DLS is a well-known tool for determining the hydrodynamic diameter of colloidal particles. A Malvern ZetaSizer Nano ZS Instrument (Malvern Instruments Ltd, UK) was used, operating at a light-source wavelength of 532 nm and a fixed scattering angle of 173° for detection. Zeta potential (ZP) measurements were also performed to study the surface properties and any changes in the surface after the exposure experiments. ZP is a useful technique to study stability of nanoparticles and their surface charge when they are electrostatically stabilized. X-ray diffraction spectra (using a PANalytical X´Pert diffractometer fitted with a Cu Kα radiation source) were also recorded to determine the crystalline phase of the samples. UV-Visible and XRD spectra for the Au, Ag, CeO_2, and TiO_2 nanoparticles used in this work are presented in Figure 1. Transmission electron microscope (TEM, using a JEOL 1010 operating at an accelerating voltage of 80 kV) images of the samples are also shown.

In all cases the nanoparticles responded similarly as with the other techniques. During and after the experiments none of the nanoparticles showed aggregation, dilution, or sedimentation, as assessed by counting the number of nanoparticles in a predetermined area by analyzing at least 50 TEM images in which nanoparticles appeared in suspension, neither sedimented nor aggregated, and in a number similar to that of initial samples (Table 1). The only nanoparticles that were not in solution were those adsorbed on the biomass; Figure 2 shows an example of this phenomenon. The TEM studies showed no morphological change in the NPs conformation.

Nanoparticle concentration in solution before and after treatment was measured taking into account the real nanoparticle mass (by inductively coupled plasma mass

spectrometry, ICPMS) of the supernatant and the pellet after NP precipitation) and the size distribution obtained by TEM.

2.3. Respirometric experiments

2.3.1. Ordinary Heterotrophic Organisms (OHO)

An enriched OHO sludge was obtained from a municipal wastewater treatment plant (Montornés del Vallès, Barcelona, Spain). The respirometer used was of a liquid-static-static (LSS) type, in which dissolved oxygen (DO) is measured in a static and non-aerated liquid phase [30].

For each respirometric test, 500 mL of OHO sludge, with an average concentration of volatile suspended solids (VSS) of 1700±560 mg VSS L^{-1}, was aerated and stirred overnight to ensure that all the substrate present in the OHO sludge was consumed. Respiration tests were performed at 25ºC. Aeration was then stopped and the DO decrease, without external substrate addition, was measured for 10 minutes using an oxygen meter (Lutron 5510, Lutron Co. Ltd., Taiwan) connected to a PC. This procedure was repeated three times and the average of the slope of the DO decrease was taken as the endogenous oxygen uptake rate (OUR_{end} in mg O_2 g^{-1} VSS h^{-1}). Afterwards, aeration was reinitiated and a pulse (30 mg mL^{-1}) of readily biodegradable chemical oxygen demand in the form of sodium acetate was added. The procedure was repeated three times to calculate the average OUR. The exogenous OUR (OUR_{ex}) was obtained by subtracting the previously determined OUR_{end} from the OUR value obtained from the available substrate. The OUR reported in this work is the OUR_{ex}. Finally, the biomass was left to settle for 1 hour and the upper portion (375 mL) was removed and substituted with the corresponding nanoparticle suspension. The loss of biomass and specific biological activity during this procedure was negligible when measured by

overall respiration [31]. The whole experimental procedure was repeated in the presence of stabilized nanoparticles to obtain the OUR_{NPs} and in the presence of the stabilizer solution alone (HMT, sodium citrate and TMAOH) to obtain the OUR_{stb}. The inhibition percentage was calculated as the reduction in OUR, with and without nanoparticles or stabilizer, and after 1 and 4 hours exposure time. This time is approximately the range of hydraulic residence time (HRT) of the biological reactors in a municipal WWTP. No significant changes in pH value were detected throughout the experimental procedure.

2.3.2. Ammonia-Oxidizing Bacteria (AOB)

An enriched AOB sludge was obtained from a partial nitrification pilot plant that had worked in continuous mode for more than five years [32]. By following the procedure described in ref. [33] for fluorescence *in situ* hybridization (FISH) analysis, the AOB population in this study accounted for 81±8% of the total biomass, whereas nitrite-oxidizing bacteria (NOB) accounted for less than 1%.

The respirometer used was a liquid-flow-static (LFS) type, where DO is measured in the liquid phase which was previously static and continuously aerated [30]. The vessel (1 L) was magnetically stirred and air flowed through a pressure manoreductor and a mass-flow controller (Bronckhorst HiTec 825) to ensure a constant airflow. The temperature of the vessel was controlled at 30±0.5ºC with a thermostatic bath. The pH was continuously measured with a pH probe (WTW Sentix 81) and controlled at 8.3±0.1 by automatic addition of acid or base by an automatic microburette (Crison Multiburette 2S). DO was measured with a DO probe (WTW-CellOx 325). Both probes were connected to multiparametric equipment (WTW-Inolab 3), which was connected via an RS232 interface to a PC that monitored the data and stored them in a Microsoft Excel worksheet through Visual Basic® 6.0 software.

The average concentration of biomass in the respirometric tests was 900±200 mg VSS L^{-1}. The OUR reported in this work corresponded to OUR_{ex} obtained by subtracting the OUR_{end} value from the total measured OUR. A detailed description of the procedure for the OUR calculation using a liquid-phase, flowing gas, static liquid (LFS) respirometer for AOB can be found elsewhere [31]. Each OUR value was corrected for possible oxygen-limitation effects by using the oxygen affinity coefficient for AOB ($K_{O,AOB}$ = 0.74 mg O_2 L^{-1} [34]).

The biomass was aerated overnight to ensure that all the substrate present in the AOB sludge was consumed. Then, the experiment started with the determination of the OUR_{end} and the oxygen transfer coefficient (k_La) following the procedure described in ref. [31]. Afterwards, a pulse of 50 mg N/L of ammonium chloride was added in the absence of nanoparticles and nanoparticle stabilizers to determine the maximum OUR (OUR_{max}). The added nitrogen was completely consumed within a few minutes and the same pulse was repeated at 1 and 4 hours. The complete procedure was repeated in the presence of nanoparticles to obtain the OUR_{NPs} and in the presence of nanoparticle stabilizers to obtain the OUR_{stb}. The percentage inhibition was calculated as the reduction of OUR_{max} with and without nanoparticles or stabilizer after 1 and 4 hours exposure to nanoparticles and stabilizers. Again, this time range was considered similar to that of the HRT of the biological reactors in a municipal WWTP.

In the OHO and AOB respirometric assays, EC_{50} was defined as the concentration of nanoparticles that causes an inhibition effect of 50%. Again, the nanoparticle concentrations were selected to determine an approximate value of EC_{50}.

2.4. Anaerobic experiments

Anaerobic inhibition tests were performed according to refs. [10,35]. Briefly, anaerobic assays were performed in 1000-mL gas-tight reactors, equipped with a pressure transducer to monitor biogas production [36]. Each anaerobic reactor contained: 250 mL inoculum (VSS=13200 ± 3000 mg L^{-1}), 250 mL sample (stabilizer or nanoparticle suspension), and 1 g cellulose as substrate, to a final volume of 500 mL. The pH value of each reactor was adjusted with sodium citrate to 8 (if necessary) and nitrogen gas was used to purge oxygen prior to incubation at 37ºC (mesophilic conditions) or 55ºC (thermophilic conditions) over approximately 50 days. Reactors were manually stirred and biogas was purged every work-day. A blank and a reference test were also performed. The blank test (250 mL of inoculum and water to 500 mL) was performed to enable biogas production from any biodegradable organic matter contained in the inoculum to be subtracted. The control test (250 mL of inoculum, 1 g of microcrystalline cellulose, and water to 500 mL) was performed to allow the comparison of the biogas production with sample (nanoparticles or stabilizers) tests. Each experiment was carried out in triplicate. The results are shown as the average value with standard deviation. Sludge for inoculation of anaerobic experiments was obtained from mesophilic and thermophilic anaerobic reactors in existing wastewater-treatment plants in the province of Barcelona. Sludge was obtained from the recirculation of these reactors. This sludge was maintained for two weeks at 37ºC or 55ºC to remove any biodegradable organic matter that could interfere in the experiments [37].

2.5. Statistical methods

An ANOVA test was performed to compare different replications under the same conditions. If the ANOVA test resulted in statistically significant differences, a

Tukey test was performed in pairwise comparisons. A 95% confidence level was selected for all statistical comparisons. Statistical tests were conducted with SPSS 15.0.1 (SPSS Inc., USA).

2.6. Routine analysis

Routine analyses such as those for volatile solids (VS), volatile suspended solids (VSS), or chemical oxygen demand (COD) were performed according to the standard procedures [37].

3. Results and discussion

3.1. Inhibition tests on OHO biomass

Table 2 shows the results of microbial activity inhibition obtained for the studied nanoparticles. In control experiments, the stabilizer solutions had no significant effect on the OHO sludge, except the TiO_2-NPs stabilizer solution (10 mM TMAOH), which showed an important inhibition (83% at 4 hours of exposure). However, this effect was not detected in the presence of TiO_2-NPs (2% inhibition at 4 hours exposure). It is probable that the presence of TiO_2-NPs provokes stabilizer sequestration, lowering its effective concentration. Similar effects on serum depletion by NPs have been reported [29]. TiO_2-NPs were synthesized at a concentration of 10^{16} NPs mL^{-1} (10^{-4} M) with a concentration of 10 mM of TMAOH used as stabilizer. Theoretically, and assuming 0.2 nm^2 as the footprint of a TMAOH molecule [38] on the surface of the particle (the ion and the counter-ion), a single nanoparticle might accommodate around 885 TMAOH molecules, which would thus decrease the free TMAOH concentration by two orders of magnitude. This assumption also recalls the use of nanoparticles to remove toxins from polluted water to make it drinkable [1]. In this case, no inhibition was observed for

TiO_2-NPs, contrary to what was found with other organisms such as *Daphnia magna* [39] and, particularly, in chronic toxicity tests [8]. The different behavior could be attributed to details in the material preparation, chemical and colloidal stability of the NPs, and presence of different additives.

No inhibition was observed for Au-NPs, whereas Ag-NPs provoked 33% inhibition after 4 hours of exposure. Other studies have reported no inhibition by Ag-NPs and Au-NPs with other toxicity tests, such as germination (at an Ag-NPs concentration of 0.1 mg mL^{-1}) or Microtox® (at an Ag-NPs concentration of 0.045 mg mL^{-1}) tests [10]. However, the growth of an OHO-type bacterium (*Pseudomonas fluorescens*) decreased 60% with 0.002 mg mL^{-1} of Ag-NPs and an exposure time of 3 hours [23]. The discrepancy with the results obtained in this work could also be due to differences in the studied microbial populations or/and to the characteristics of the Ag-NPs solutions used in both studies. Some Ag NP synthesis recipes do not reduce all the silver ions [40]; therefore the presence of a significant level of Ag^+ ions could be responsible for the observed effects in other studies. In the present study, we hypothesize that only a small proportion of the silver ions could have been dissolved, as nanoparticles mostly remain unchanged in solution, which was confirmed by TEM quantification of the silver nanoparticles. Moreover, the presence of sodium citrate in the solution may complex and therefore detoxify any ion coming from the synthesis or leached from the nanoparticles.

The case of CeO_2-NPs is the most relevant in terms of inhibition. In Figure 3, the results after 1 and 4 hours of exposure are presented. It is evident that CeO_2-NPs present the highest level of inhibition of all the studied nanoparticles. Nevertheless, we hypothesize that the microbial population has some capacity to adapt to these NPs, since

the results after four hours of exposure show a slightly lower level of inhibition than initially (Figure 3). The EC_{50} values can be estimated from Figure 3 to be 0.18 and 0.28 mg mL^{-1} for 1 and 4 hours of exposure, respectively, which might confirm this hypothesis, in the absence of more data related to the microorganism-nanoparticle interaction. On the contrary, an OHO biomass from a municipal WWTP in Switzerland was not affected by 1 mg mL^{-1} of CeO_2-NPs [19]. Similar to the case for Ag-NPs, this discrepancy could be related to differences in the characteristics of the bacterial community and the nanoparticles used in both studies. Depending on the synthetic route used to produce the NPs, a difference in the number of oxygen vacancies will be present in the CeO_2-NP, which will promote their catalytic activity as oxygen sponges [41]. Interestingly, it seems that while the TiO_2-NPs are able to decrease the toxicity of TMAOH, addition of the non-toxic HMT solution is not able to prevent the toxicity of the CeO_2 nanoparticles, which indicates the importance of the interaction between nanoparticles and additives [10].

3.2. Inhibition tests on AOB biomass

Table 3 shows the results of the inhibition tests on the studied nanoparticles with an enriched AOB population. As mentioned above, nanoparticle stabilizer solutions had a slight inhibitory effect on the AOB biomass (between 2 and 14%), with the TiO_2-NPs stabilizer (10 mM TMAOH) effect being the most important. Again, the inhibitory effects of Ag-NPs and TiO_2-NPs stabilizers were higher than the inhibition caused by Ag-NPs and TiO_2-NPs (lower than 4%), which is similar to the results obtained with TiO_2-NPs and OHO biomass. Indeed, the inhibitory effect of Ag-NPs and TiO_2-NPs on AOB biomass was not significant. The case of Ag-NPs is especially important because it has been deeply studied by other authors [20-22,24], who found a great inhibitory

effect of Ag-NPs on nitrifying biomass and suggested that Ag-NPs were more toxic to nitrifying bacteria than were Ag ions (Ag^+). Choi et al. [20] reported, at the same concentration, 20% of inhibition on a nitrifying suspension. Ag-NPs used in the work reported in ref. [20] had an average diameter of 14 nm, while in the present study the nanoparticles are 30 nm (mean diameter). Thus, this discrepancy could be due to the different characteristics of the Ag-NPs in both studies. In fact, Choi et al. [20] reported the importance of the nanoparticle diameter when assaying the toxicity of Ag-NPs on nitrifying biomass, concluding that smaller nanoparticles cause a greater inhibition effect. However, this result could be also explained because when comparing sizes, for the same mass, the nanoparticle concentration exponentially increases as their diameter decreases. In fact, doses can only be meaningfully compared when normalized to surface area or number of particles. In addition, it should be remembered that some Ag^+ ions remain in every synthesis. In some of the commercial samples of colloidal silver intended for water purification, the amount of ionic silver may be as high as 90% with respect to the total silver content. This fact might also have an important impact on the toxicity of Ag-NPs. In the case of Au-NPs, in contrast to OHO biomass, an inhibitory effect (around 14%) was detected, but it was low and it did not increase with exposure time.

As detected with OHO biomass, the case of CeO_2-NPs is the most relevant in terms of inhibition for AOB biomass. In Figure 4, the results after 1 and 4 hours of exposure are presented. The EC_{50} values can be estimated from Figure 4 to be 0.21 and 0.05 mg mL^{-1} after 1 and 4 hours, respectively, which shows that exposure time was a crucial factor when dealing with inhibition by CeO_2-NPs. Other authors found the

inhibition by zero-valent nanoparticles of other bacterial populations to be dependent on time [42].

3.3. Inhibition test on anaerobic consortia

Anaerobic biogas production tests were carried out for the nanoparticles studied in the presence of mesophilic and thermophilic communities of anaerobic populations obtained from large-scale anaerobic digesters. Microcrystalline cellulose was used as the sole substrate for anaerobic digestion as it requires the participation of all the microbial communities involved in the anaerobic processing of organic matter [10]. The results obtained for mesophilic and thermophilic populations are presented in Figure 5. In this case, the contribution of stabilizers was negligible; with no observable toxicity. Furthermore, the biogas production was not statistically different to that of control experiments where no nanoparticles were present and the substrate for anaerobic digestion was also cellulose (data not shown).

Statistical analysis of the data in Figure 5 gives some information about the influence of certain nanoparticles on the anaerobic consortia. No statistical differences were found among all the experiments studied under either mesophilic or thermophilic conditions, except for in the cases of TiO_2 and CeO_2 nanoparticles. In the case of TiO_2-NPs, a slight positive effect on the production of biogas (10% increase, $p<0.05$) was detected in the thermophilic anaerobic test. CeO_2-NPs again caused a drastic inhibition (90%) in both mesophilic and thermophilic anaerobic consortia, which is characterized by a significant reduction of biogas production. In the case of CeO_2-NPs, dilutions were carried out to determine the EC_{50} value for mesophilic populations. CeO_2-NPs did not have toxicity effects on mesophilic anaerobic biomass at concentrations under 0.16 mg mL^{-1}, and the measured EC_{50} value was 0.26 mg mL^{-1}. From the experiment performed

with thermophilic anaerobic consortia, it can be deduced that this EC_{50} value will be lower than 0.32 mg mL^{-1}.

It is important to mention that no data on the toxicological effects of nanoparticles on an anaerobic population have been found in the literature to compare with the results obtained in this work, except a study that showed no toxicity in mesophilic anaerobic populations exposed to fullerenes [43]. Clearly, the chemical structure of fullerenes is completely different to that of the inorganic nanoparticles used in our study.

3.4. Effects of properties and doses of NPs on toxicity

In the light of the results and discrepancies obtained in this and other works with Ag and CeO_2-NPs for both OHO and AOB biomasses, it is evident that when reporting the toxicity effects of nanoparticles it is essential to describe the characterization parameters (size, surface charge, presence of stabilizers, etc.) and the possible changes in nanoparticles throughout process [44]. In this context, it is important to recall the unstable nature of colloids. This instability favors their aggregation and sedimentation as soon as they are extracted from the environment in which they were synthesized. Aggregation leads to sedimentation and may induce both false negatives (due to the nanoparticles not participating in the experiment) and false positives (microparticles resulting from the aggregation of the nanoparticles may show a different and increased toxicity profile [45]). This fact supports the inclusion of the effect of the stabilizers to prevent agglomeration in studies on nanotoxicology. In addition, together with the nanoparticles, residues from the synthesis are often present in the form of metal ions if the samples are not completely purified after synthesis; these could interfere with the nanoparticles. Coupled with this problem there is the issue of how to determine of

realistic doses. Firstly, colloids of inorganic nanoparticles in suspension are systems out of equilibrium and are difficult to prepare at any desired concentration, since at high concentrations the nanoparticles can be removed from the solution phase when a saturation concentration is reached, by an entropy effect similar to that seen in salts (at high concentrations, nanoparticles can constantly collide in solution, resulting in their precipitation). These saturation concentrations are between the micro- and the millimolar values, depending on the material and the preparation procedure. Therefore, the concentrations expected to be found in real cases (e.g., inside the body or in the environment) would normally be more dilute than the prepared samples, unless accumulation occurs. What is clear is that a large number of physicochemical parameters will have a strong influence on the toxicity of nanoparticles and on the methods used to evaluate it. The case of CeO_2 deserves special attention as it is applied as a catalytic converter in the automotive industry for the reduction of toxic emissions from internal combustion engines, as antioxidant in biomedicine to treat disorders caused by oxygen radicals, as an additive in fuel cells, and as a UV absorber, among many other applications. All these applications seem to rely on the capability of CeO_2 to store or release oxygen, depending on the surrounding conditions. This capability depends on the crystal structure which, at the same time, depends on the synthesis process. Apparently, the catalytic properties may perturb the respiration mechanisms of the studied microbial communities, which leads to the observed inhibition. It is also worth noting that these particles did not show toxicity in mammalian cells [29], which is probably due to the more robust structure and better defense mechanism of eukaryote versus prokaryote cells, although very recent studies have reported toxicity of cerium oxide nanoparticles on an eukaryote cell line, specifically DNA damage [46].

In the case of Ag-NPs, the low toxicity found in this study compared to previous literature [47] can be explained since, recently, it has been demonstrated that in a WWTP most of the Ag in the sludge and the effluent was present in the form of Ag_2S, which is less toxic than free Ag [48]. This hypothesis is, however, untested in the reported experiments. Moreover, to the best of our knowledge, this is the only published paper performed in similar conditions (using a pilot-scale WWTP) to those used in our study.

Even if the aim of this work was not to compare toxicity between different NPs but to assess the toxicity of the common ones, it has to be noted that the highest concentration and surface area of TiO_2 does not correspond to an increased toxicity, while gold and silver show some inhibition capacities at much lower values of number and surface area. This result is consistent with the normal production of both materials; the oxides in large quantities and the precious metals in smaller ones. The differences in toxicity should be attributed to composition and not size, since size-dependent biological effects of inorganic nanoparticles have been observed where larger size differences exist [49].

5. Conclusions

In the present work, respiration tests and biogas production were used to evaluate the effect of four different metal oxide (CeO_2 and TiO_2) and zero-valent metal (Ag and Au) nanoparticles (NPs) on the activity of the most important microbial communities of a wastewater treatment plant (WWTP); ordinary heterotrophic organisms (OHO), ammonia oxidizing bacteria (AOB), and thermophilic and mesophilic anaerobic bacteria.

Au-NPs and TiO_2-NPs obtained with the characteristics reported in this study present zero or low toxicity towards OHO, AOB, and anaerobic biomass, while Ag-NPs present an intermediate toxicity (inhibition around 33% on OHO at a concentration of 0.13 mg mL^{-1} and an exposure time of 4 hours) and CeO_2-NPs were the most toxic (1 hour exposure: OHO-EC_{50}=0.18 mg mL^{-1} and AOB-EC_{50}=0.21 mg mL^{-1}, respectively; 4 hours exposure: OHO-EC_{50}=0.28 mg mL^{-1} and AOB-EC_{50}=0.05 mg mL^{-1}, respectively; EC_{50} of CeO_2-NPs for mesophilic anaerobic bacteria was calculated to be around 0.26 mg mL^{-1}, while it was lower than 0.32 mg mL^{-1} for the thermophilic ones). It has to be noted, however, that the concentrations assayed in this study are likely much higher than those that would be expected in a municipal WWTP and the susceptibility of those communities to NP formulation varies. As future work, the study of the effect of nanoparticles and the stabilizers used for environmental applications on the specific species present in wastewater would be of great interest as well as the real solubility/availability of NPs in several wastewaters. The effect of stabilizers on the catalytic properties of nanoparticles should also be studied.

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References

[1] C.T. Yavuz, J.T. Mayo, W.W. Yu, A. Prakash, J.C. Falkner, S. Yean, L. Cong, H.J. Shipley, A. Kan, M. Tomson, D. Natelson, V.L. Colvin, Low-field magnetic separation of monodisperse Fe_3O_4 nanocrystals, Science 314 (2006) 964-967.

[2] S. Recillas, J. Colón, E. Casals, E. González, V. Puntes, A. Sánchez, X. Font, Chromium VI adsorption on cerium oxide nanoparticles and morphology changes during the process, J. Hazard. Mat. 184 (2010) 425-431.

[3] M.N. Moore, So nanoparticles present ecotoxicological risks for the health of the aquatic environment?, Environ. Int. 32 (2006) 967-976.

[4] S.K. Brar, M. Verma, R.D. Tyagi, R.Y. Surampalli, Engineered nanoparticles in wastewater sludge – Evidence and impacts, Waste Manage. 30 (2010) 504-520.

[5] A. Franco, A.F. Hansen, S.I. Olsen, L. Butti, Limits and prospects of the incremental approach on the European legislation on the management of risk related to nanomaterials, Regul. Toxicol. Pharm. 48 (2007) 171-183.

[6] A. Sánchez, S. Recillas, X, Font, E. Casals, E. Gonzalez, V. Puntes, Engineered inorganic nanoparticles and the environment: Remediation and Ecotoxicity, TRAC 30 (2011) 507-516.

[7] R. Kaegi, A. Ulrich, B. Sinnet, R. Vonback, A. Wichser, S. Zuleeg, H. Simmler, S. Brunner, H. Vonmont, M. Burkhardt, M. Boller, Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment, Environ. Pollut. 156 (2008) 233-239.

[8] X. Zhu, Y. Chang, Y. Chen, Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*, Chemosphere 78 (2010) 209-215.

[9] D. Lin, B. Xing, Phytotoxicity of nanoparticles: inhibition of seed germination and root growth, Environ. Pollut. 150 (2007) 243-250.

[10] R. Barrena, E. Casals, J. Colón, X. Font, A. Sánchez, V. Puntes, Evaluation of the ecotoxicity of model nanoparticles, Chemosphere 75 (2009) 850-857.

[11] A. Kahru, H.C. Dubourguier, I. Blinova, A. Ivask, K. Kasemets, Biotests and biosensors for ecotoxicology of metal oxide nanoparticles: A minireview, Sensors 8 (2008) 5153-5170.

[12] A. García, R. Espinosa, L. Delgado, E. Casals, E. González, V. Puntes, C. Barata, X. Font, A. Sánchez, Acute toxicity of cerium oxide, titanium oxide and iron oxide nanoparticles using standardized tests, Desalination 269 (2011) 136-141.

[13] S.A. Blaser, M. Scheringer, A. MacLeod, K. Hungerbühler, Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles, Sci. Total Environ. 390 (2010) 396-409.

[14] M.J. Osmond, M.J. Mccall, Zinc oxide nanoparticles in modern sunscreens: An analysis of potential exposure and hazard, Nanotoxicol. 4 (2010) 15-41.

[15] T.M. Been, P. Westerhoff, Nanoparticle silver released into water from commercially available sock fabrics, Environ. Sci. Technol. 42 (2008) 4133-4139.

[16] F. Gottschalk, T. Sonderer, R.W. Scholz, B. Nowack, Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions, Environ. Sci. Technol. 43 (2009) 9216-9222.

[17] M.A. Kiser, P. Westerhoff, T. Benn, Y. Wang, J. Pérez-Rivera, K. Hristovski, Titanium nanomaterial removal and release from wastewater treatment plants, Environ. Sci. Technol. 43 (2009) 6757-6763.

[18] B. Kim, C.S. Park, M. Murayama, M.F. Hochella, Discovery and characterization of silver sulfide nanoparticles in final sewage sludge products, Environ. Sci. Technol. 44 (2010) 7509-7514.

[19] L. Limbach, R. Bereiter, E. Müller, R. Krebs, R. Gälli, W. Stark, Removal of oxide nanoparticles in a model wastewater treatment plant: influence of agglomeration and surfactants on clearing efficiency, Environ. Sci. Technol. 42 (2008) 5828-5833.

[20] O.K. Choi, K.K. Deng, N.J. Kim, L. Ross Jr., R.Y Surampalli, Z.Q. Hu, The inhibitory effect of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth, Water Res. 42 (2008) 3066-3074.

[21] O.K. Choi, Z.Q. Hu, Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria, Environ. Sci. Technol. 42 (2008) 4583-4588.

[22] O.K. Choi, Z.Q. Hu, Nitrification inhibition by silver nanoparticles, Water Sci. Technol. 59 (2009) 1699-1702.

[23] J. Fabrega, S.R. Fawcett, J.C. Renshaw, J.R. Lead, Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter, Environ. Sci. Technol. 43 (2009) 7285-7290.

[24] Z. Liang, A. Das, Z.Q. Hu, Bacterial response to a shock load of nanosilver in an activated sludge treatment system, Water Res. 44 (2010) 5432-5438.

[25] F. Zhang, Q. Jin, S.W. Chan, Ceria nanoparticles: Size, size distribution, and shape, J. Appl. Phys. 95 (2004) 4319-4326.

[26] A. Pottier, S. Cassaignon, C. Chaneac, F. Villain, E. Tronca, J.P. Jolivet, Size tailoring of TiO_2 anatase nanoparticles in aqueous medium and synthesis of nanocomposites, Characterization by Raman spectroscopy. J. Mater. Chem. 13 (2003) 877-882.

[27] J. Turkevich, P.C. Stevenson, J. Hillier, Nucleation and growth process in the synthesis of colloidal gold, J. Discuss. Faraday Soc. 11 (1951) 55.

[28] J.F. Berret, A. Sehgal, M. Morvan, O. Sandre, A. Vacher, M. Airiau, Stable oxide nanoparticle clusters obtained by complexation, J. Colloid Interface Sci. 303 (2006) 315-318.

[29] T. Pfaller, V. Puntes, E. Casals, A. Duschl, G.J. Oostingh, In vitro investigation of immunomodulatory effects caused by engineered inorganic nanoparticles - the impact of experimental design and cell choice, Nanotoxicol. 3 (2009) 46-59.

[30] H. Spanjers, P. Vanrolleghem, G. Olsson, P.L. Dold, Respirometry in control of the activated sludge process: principles, Scientific and technical report no. 7, IWA Publishing, London, 1998.

[31] A. Guisasola, J.A. Baeza, J. Carrera, C. Casas, J. Lafuente, An off-line respirometric procedure to determine inhibition and toxicity of biodegradable compounds in biomass from an industrial WWTP, Water Sci. Technol. 48 (2003) 267-275.

[32] I. Jubany, J. Lafuente, J.A. Baeza, J. Carrera, Total and stable washout of nitrite oxidizing bacteria from a nitrifying continuous activated sludge system using

automatic control based on Oxygen Uptake Rate measurements, Water Res. 43 (2009) 2761-2772.

[33] I. Jubany, J. Lafuente, J. Carrera, J.A. Baeza, Automated thresholding method (ATM) for biomass fraction determination using FISH and confocal microscopy, J. Chem. Technol. Biotechnol. 84 (2009) 1140-1145.

[34] A. Guisasola, I. Jubany, J.A. Baeza, J. Carrera, J. Lafuente, Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation, J. Chem. Technol. Biotechnol. 80 (2005) 388-396.

[35] A. Schievano, M. Pognani, G. D'Imporzano, F. Adani, Predicting anaerobic biogasification potential of ingestates and digestates of a full-scale biogas plant using chemical and biological parameters, Bioresource Technol. 99 (2008) 8112-8117.

[36] R. Barrena, G. d'Imporzano, S. Ponsá, T. Gea, A. Artola, F. Vázquez, A. Sánchez, F. Adani, In search of a reliable technique for the determination of the biological stability of the organic matter in the mechanical-biological treated waste, J. Hazard. Mat. 162 (2009) 1065-1072.

[37] APHA, Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, Washington, USA, 1998.

[38] H.A. Biebuyck, G.M. Whitesides, Self-Organization of Organic Liquids on Patterned Self-Assembled Monolayers of Alkanethiolates on Gold, Langmuir 10 (1994) 2790-2793.

[39] S.B. Lovern, R. Klaper, *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C_{60}) nanoparticles, Environ. Toxicol. Chem. 25 (2006) 1132-1137.

[40] E. Navarro, F. Piccapietra, B. Wagner, F. Marconi, R. Kaegi, N. Odzak, L. Sigg, R. Behra, Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*, Environ. Sci. Technol. 42 (2008) 8959-8964.

[41] A.S. Karakoti, N.A. Monteiro-Riviere, R. Aggarwal, J.P. Davis, R.J. Narayan, W.T. Self, J. McGinnis, S. Seal, Nanoceria as antioxidant: Synthesis and biomedical applications, J.O.M. 60 (2008) 33-37.

[42] F. Rispoli, A. Angelov, D. Badia, A. Kumar, S. Seal, V. Shah, Understanding the toxicity of aggregated zero valent copper nanoparticles against *Escherichia coli*, J. Hazard. Mat. 15 (2010) 212-216.

[43] L. Nyberg, R.F. Turco, L. Nies, Assessing the Impact of Nanomaterials on Anaerobic Microbial Communities, Environ. Sci. Technol. 42 (2008) 1938-1943.

[44] D.B. Warheit, Debunking Some Misconceptions about Nanotoxicology, Nano Lett. 10 (2010) 4777-4782.

[45] C.A. Poland, R. Duffin, I. Kinloch, A. Maynard, W.A.H. Wallace, A. Seaton, V. Stone, S. Brown, W. MacNee, K. Donaldson, Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat. Nanotechnol. 3 (2008) 423-428.

[46] M. Auffan, J. Rose, T. Orsiere, M. De Meo, A. Thill, O. Zeyons, O. Proux, A. Masion, P. Chaurand, O. Spalla, A. Botta, M.R. Wiesner, J.Y. Bottero, CeO_2 nanoparticles induce DNA damage towards human dermal fibroblasts in vitro, Nanotoxicol. 3 (2009) 161-171.

[47] N. Kumar, V. Shah, V.K. Walker VK, Perturbation of an arctic soil microbial community by metal nanoparticles, J. Hazard. Mat. 190 (2011) 816-822.

[48] R. Kaegi, A. Voegelin, B. Sinnet, S. Zuleeg, H. Hagendorfer, M. Burkhardt, H. Siegrist, Behavior of Metallic Silver Nanoparticles in a Pilot Wastewater Treatment Plant, Environ. Sci. Technol. 45 (2011) 3902-3908.

[49] B.D. Chithrani, A.A. Ghazani, W.C. Chan, Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells, Nano Lett. 6 (2006) 662-668.

Tables

Table 1. Main characteristics of the nanoparticles used (concentrations of nanoparticles were obtained as they were synthesized).

Nanoparticle	**CeO_2**	**TiO_2**	**Au**	**Ag**
Concentration (mg/mL)	0.64	1.12	0.10	0.17
Approximate number of NPs (NPs/mL)	~10^{16}	~10^{16}	~10^{13}	~10^{12}
Mean size (nm)	12	7.5	20	30
Shape	spherical	spherical	spherical	spherical
Zeta potential (mV)	+11.5	-42.5	-44.3	-39.2
Stabilizer*	HMT	TMAOH	S. Citr.	S. Citr.
Stabilizer concentration (mM)	8.3	10	0.89	10
pH (original)	9	9	8.5	8.5
Estimated surface area (m^2/g)	121	186	16	19

*HMT: Hexamethylene tetramine; TMAOH: Tetramethyl ammonium hydroxide; S. Citr.: Sodium citrate.

Table 2. Inhibition of OHO by NPs and stabilizers.

Nanoparticles	**CeO_2**		**TiO_2**		**Au**		**Ag**	
Concentration (mg/mL)	0.64		0.84		0.075		0.13	
Exposure time (h)	1	4	1	4	1	4	1	4
Inhibition of NPs (%)	100	100	1	2	0	7	0	33
Inhibition of NPs stabilizer (%)	0	0	0	83	0	3	0	8

Table 3. Inhibition of AOB by NPs and stabilizers after different exposure times.

Nanoparticles	**Au**		**Ag**				**TiO_2**						**CeO_2**									
Concentration (mg/mL)	0.09		0		0.1		0.2		0.56		1.01		0.03		0.04		0.06		0.128		0.576	
Exposure time (h)	1	4	1	4	1	4	1	4	1	4	1	4	1	4	1	4	1	4	1	4	1	4
Inhibition of NPs (%)	14	13	0	0	0	2	0	0	5	0	4	2	0	15	1	22	0	67	32	85	100	100
Inhibition of NPs stabilizer (%)	5	9	2	7	2	7	2	7	3	14	3	14	7	7	7	7	7	7	7	7	7	7

Figure Legends

Figure 1: UV-Visible spectra and XRD spectra for the Au, Ag, CeO_2, and TiO_2 nanoparticles used in this work. Scale bar in TEM images represents 100 nm.

Figure 2: Example of TEM images of anaerobic mesophilic bacterium in the presence of nanoparticles: a) Au nanoparticles, b) Ag nanoparticles, c) CeO_2 nanoparticles and d) TiO_2 nanoparticles. Scale bar in TEM images represents 200 nm for Au and Ag nanoparticles and 500 nm for CeO_2 and TiO_2 nanoparticles, respectively.

Figure 3: Inhibition of OHO by CeO_2-NPs after 1 and 4 hours of exposure. The bars are presented as an average value of a triplicate measurement with the corresponding standard deviation.

Figure 4: Inhibition of AOB by CeO_2-NPs after 1 and 4 hours of exposure. The bars are presented as an average value of a triplicate measurement with the corresponding standard deviation.

Figure 5: Biogas production (expressed as normal mL of biogas per g of initial volatile solid) of mesophilic and thermophilic anaerobic populations in the presence of the nanoparticles studied at its nominal (Table 1) concentration. The bars are presented as an average value of a triplicate measurement with the corresponding standard deviation.

Figure 1: García et al.

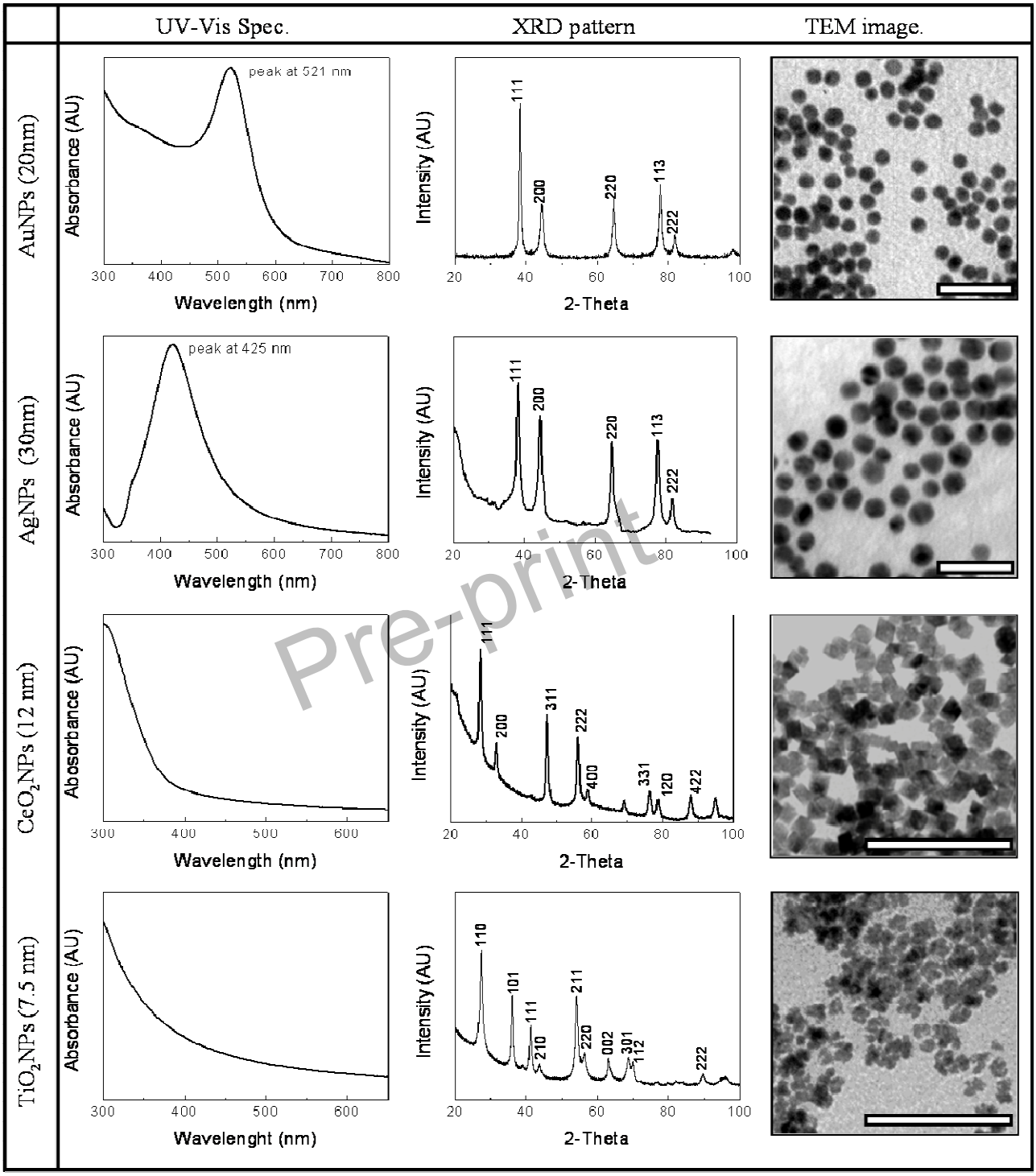
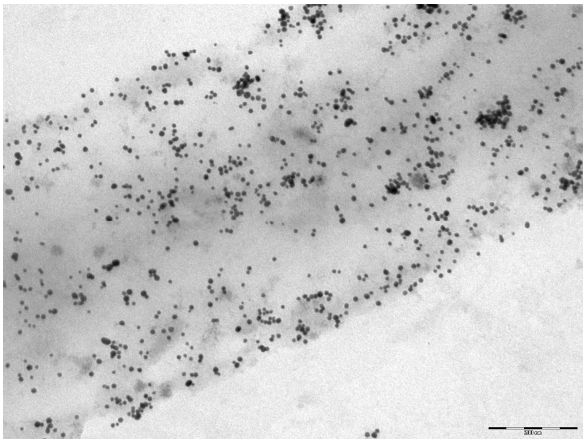


Figure 2: García et al.

a)



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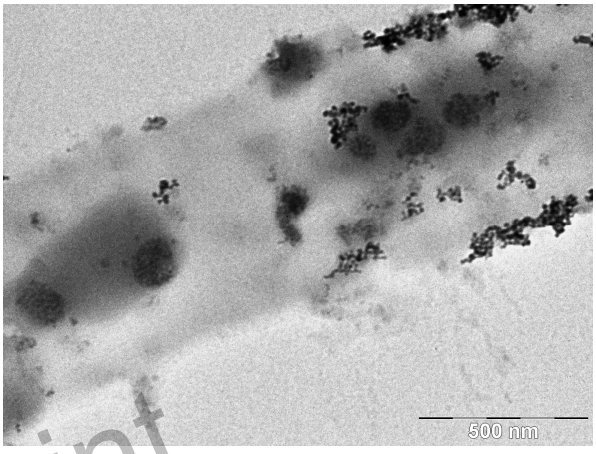

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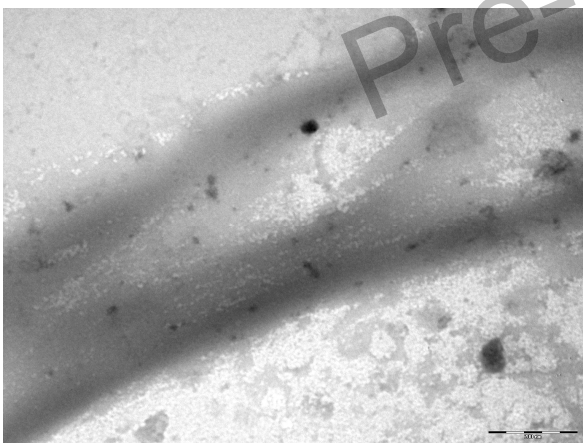

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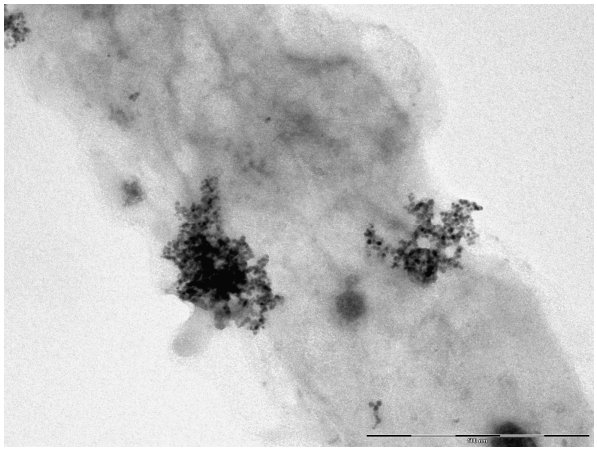

Figure 3: García et al.

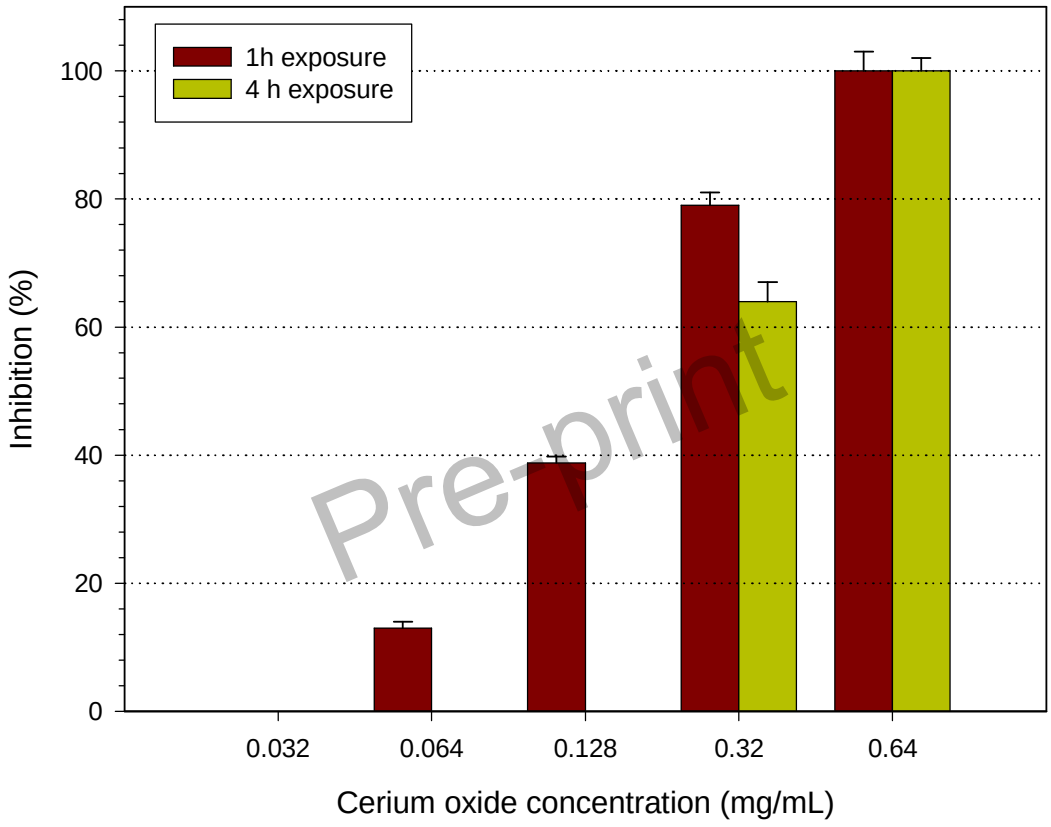

Figure 4: García et al.

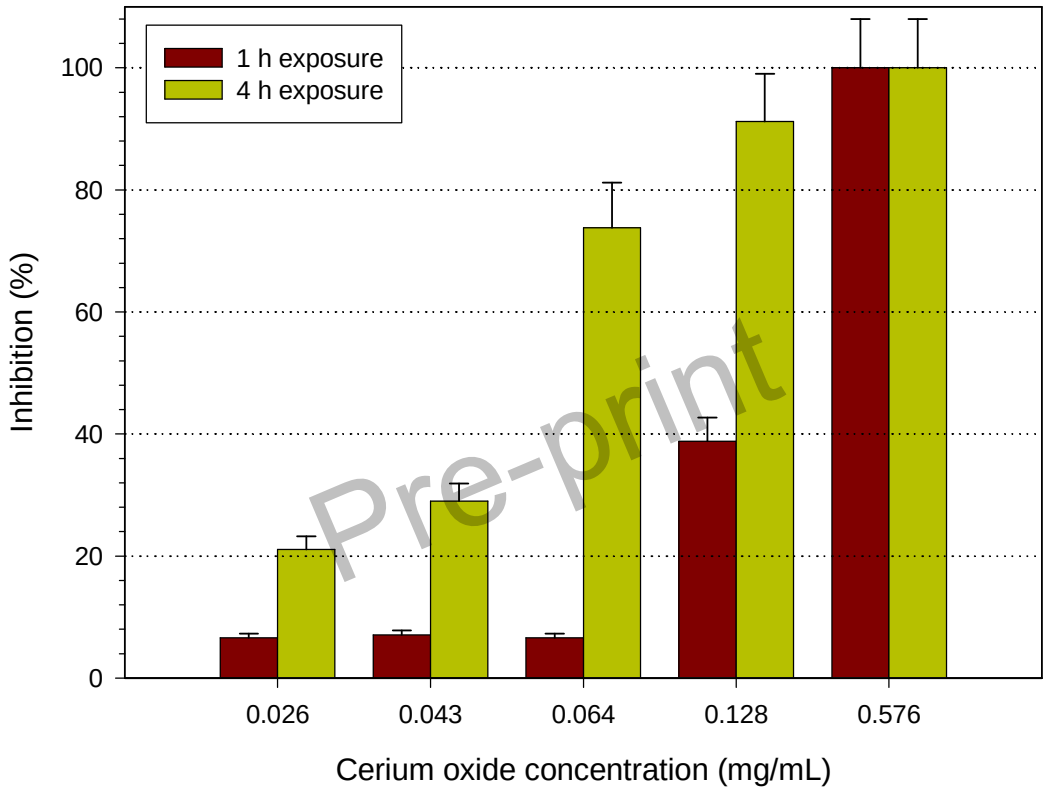

Figure 5: García et al.