Effects of Dissolved Silver and Silver Nanoparticle on Soil Microorganisms

Ezzat R. Marzouk

Soil and Water Sciences Department, Faculty of Environmental Agricultural Sciences, Arish University, North Sinai 45516, Egypt

Abstract: Silver nanoparticles (AgNPs) are the most commonly used man-made nanomaterial in consumer products. They are merged into a vast collection of products due to their particular broad-spectrum anti-microbial action. On the other hand, their anti-bacterial properties may pose a significant environmental risk. This study was undertaken to assess whether the risk that AgNPs and dissolved Ag pose to soil microorganisms gained from nano- or dissolved-silver forms. The antimicrobial effect of green manufactured AgNPs (46.2 ± 23.2 nm) and Ag^+ (as $AgNO_3$) on soil microbes were studied using disc diffusion assay. Soil microbial growth was assessed by the measurement of inhibitory zone area (mm^2) as a function of different AgNPs and $AgNO_3$ concentrations, 0.00, 250, 500, 1000 and 2000 mgl^{-1}. The results of antimicrobial effect of AgNPs showed 10 fold of magnitude comparing with Ag^+ at lower concentrations (250 mgl^{-1}). The theoretical predicted unity of inhibition zone ratio for AgNPs/Ag ions implies that AgNPs equates to about 5 times antimicrobial effects of Ag^+. Although the current results confirm that Ag antimicrobial effect is a nano-specific effect, different bacteria isolated from different soils should be used to explore their physico-chemical properties in mitigating AgNPs toxicity effects.

Keywords: Silver nanoparticles; dissolved silver; antimicrobial effects; soil microorganisms

INTRODUCTION

The advantages of new emergence of engineered nanoparticles (ENPs) have been recently recognized (Cornier *et al.*, 2017; Solaiman *et al.*, 2017). Although these ENPs have been produced for decades, extraordinary marketable production began only in recent years. Products incorporating ENPs were doubled in the last five years to reach more than 3600 consumer products (Bour *et al.*, 2015) and expected to be more grown in the future due to their potential new benefits. Presently, silver nanoparticles (AgNPs) are the most widely commercialized ENPs (Rahmatpour *et al.*, 2017; Buzea *et al.*, 2007) that are used in various applications starting from industry and ending to medications and cosmetics. It has been reported that production of AgNPs is over 400 tons per year, 30% of which are used in medical applications all over the world (Pourzahedi and Eckelman, 2014). Consequently, AgNPs will eventually be released into the soil without consideration of their potential risks on soil microorganisms during their production, use, and disposal actions (Anjum *et al.*, 2013).

Soil microorganism community depicted a large portion of global genetic diversity and are the largest group of organisms in the earth (Bot and Benites, 2005); both in terms of either the number of different species or overall biomass. Soil microbes range from 0.5 to 100 μm, in size, for bacteria and nematodes, respectively. They have a number of important functions in soil that make it as an active biosphere including: weathering processes, establishment and maintenance of soil aggregates, organic matter transformation, plant diversity and nutrients cycling (Gorbushina and Krumbein, 2005). So, the numerous roles of soil microorganisms keep the environment in a safe status and any change to their structure will defiantly affect ecological processes and ultimately the human kind.

Silver nanoparticles have been extensively studied through which it may affect soil biota in two ways; (i) effects due to particle size (nanosize), as a "nano-specific" effects and/or (ii) effects to the releasing of metal ions as Ag^+ (Dimkpa, 2014). It is very difficult, in natural systems, to determine if effects are caused by released metal ions or if they are "nano-specific" (Garcia-Reyero *et al.*, 2014). This is because a limitation of analytical methods that can distinguish between AgNPs and Ag ions. Very few studies have investigated the behaviour of AgNPs in soil - regardless of whether the effects are "nano-specific" or not - as most studies have focused on aquatic systems (Cornelis *et al.*, 2014). Given that soils are a likely ultimate sink for AgNPs, the main objective of the current work was to carry out a concise study of the toxicity effect of AgNPs comparing with Ag ions on soil microorganisms.

MATERIALS AND METHODS

Silver nanoparticles preparation

Nanoparticles of silver were purchased from Agriculture Research Centre (ARC) Giza, Egypt. The AgNPs were synthesised as described by Vigneshwaran *et al* (2006). Briefly, silver nanoparticles were produced using green chemistry method where 1.0 g of soluble starch was dissolved in 100 ml of deionized water. One ml of a 100 mM $AgNO_3$ was added and well stirred. This mixture was autoclaved at 15 psi pressure, 121ºC for 5 min. The resulting colour of the nanoparticle suspension was clear yellow indicating the formation of silver nanoparticles. Silver nanoparticles were brought from ARC at concentration of 100 mgl^{-1} and at diameter size less than 100 nm. The solution was then freeze dried and the resulting powdered AgNPs were then used to prepare different AgNPs concentrations (the AgNPs powder were re-suspended in 5 ml of distilled water resulting in a concentration of 2000 mgl^{-1} AgNPs). To check the nanosize of AgNPs provided from ARC, scanning electronic microscope (SEM; Quanta 450 FEG-ESEM, FEI Company) was used which confirm a

size of less than 100 nm (average size of 46.2 ± 23.2 nm; Figure 1). Nanoparticles have a widely agreed definition that have "characteristic dimension from 1 to 100 nm and have properties that are not shared by non-nanoscale particles with the same chemical composition" (Auffan *et al.*, 2009).
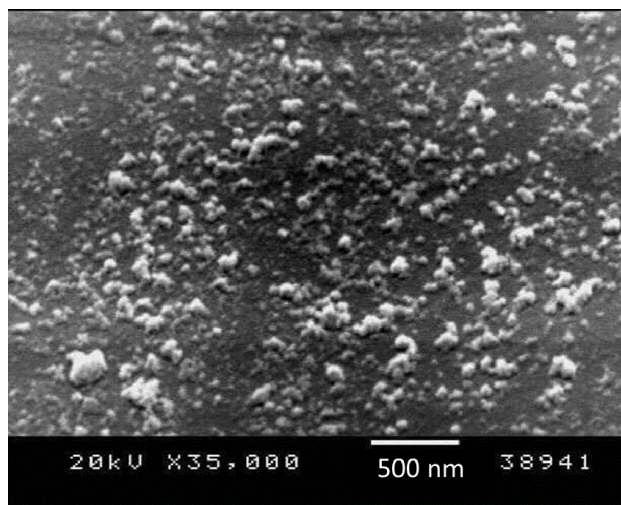

Figure (1): SEM image of silver nanoparticles that confirm the size of less than 100 nm (average of 46.2 ± 23.2 nm).

Investigation of antibacterial potency of dissolved Ag and AgNPs

The nutrient agar disc diffusion method (Perumalsamy *et al.*, 2006) was used to test the antibacterial activity of Ag of different species (AgNPs and Ag^+ ions). A soil sample was collected from cultivated field at Zagazig City, Egypt. Soil water extract was used to prepare the culture suspension in order to set an intense inoculum using serial decimal dilution method. One ml of the soil suspension (second dilution) was added to each sterile Petri dish followed by an addition of 20 ml of sterile nutrient agar. The medium was allowed to solid for 3 min and sterile paper discs (6 mm in diameter) were placed on them that have different concentrations of either AgNPs or ionic Ag. Discs were soaked in solution containing different concentrations of AgNPs and Ag^+ (as $AgNO_3$) as 250, 500, 1000 and 2000 mgl^{-1}. Although highest Ag concentrations (1000 and 2000 mgl^{-1}) are environmental irrelevant concentrations, they are necessary for modelling the complete responses of a wide diversity of soil microorganisms (see section 2.3). Therefore, the concentration range of silver was selected according to the minimum inhibitory concentration (MIC) which was reported to range from 0.02 to 65 mgl^{-1} for sensitive bacteria and up to 2675 mgl^{-1} for resistant bacteria (Miller *et al.*, 2013). Discs were soaked in 20 μl of 70% ethanol served as control for both Ag forms. The inoculated plates were incubated at 37°C for 4 days. Inhibition effects were measured in terms of inhibition zone (halo zone) around the paper disc with scale. The inhibition zone was measured from the one edge of the inhibition zone to another passing through the centre of the disc then dividing the value by two to calculate the

radius (Figure 2). This was repeated three times in the same inhibition zone and the mean radius was calculated. The area of inhibition zone was calculated using the formula:

$$A_{in} = \pi R^2 - \pi r^2 \qquad (1)$$

where A_{in} = inhibition zone area π = 3.143, R = radius of the inhibition zone and r = radius of the paper disc. The absolute mean area of inhibition produced (A_{in}) was calculated as an effect of silver applications. Various clear zones, more than 6 mm in diameter, that were observed at different concentrations of silver forms for varied concentrations were measured in millimeter and the average of the inhibition zones were recorded. All treatments were undertaken in triplicates.
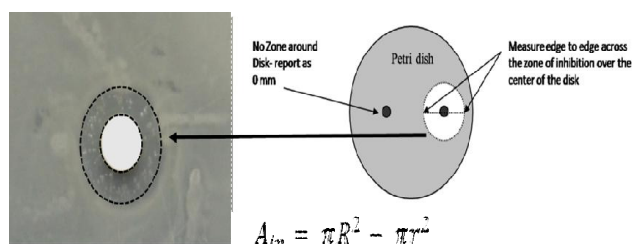

Figure (2): Measure of inhibition zone using disc diffusion methods and equation 1.

Predicting AgNPs/Ag$^+$ ratio of inhibition zone area (A_{in}) as a function of AgNPs concentrations

A power equation has been used to predict AgNPs/Ag$^+$ inhibition zone ratio (AgNPs/Ag$_{in}$) as a function of AgNPs concentrations as shown by Eq 2:

$$Y_p = a\,(X_m)^b \qquad (2)$$

where Y_p is the predicted AgNP/Ag$_{in}$, X_m is the concentration of AgNPs (mgl^{-1}), while a and b (can be positive or negative) are the constant values of the power equation. This equation was selected based on the best fitting line that produces the highest R^2 value (coefficient of determination) using Microsoft Excel 2016. Although, liner, exponential, logarithmic and polynomial equations were tested and gave reasonable R^2 values, a power equation provided the best fitting line with high R^2 value. With this equation, we aiming to predict the theoretical ratio of unity (AgNPs/Ag$_{in}$ = 1) in order to estimate the concentration of dissolved Ag that produce the same inhibition area of AgNPs. The equation was optimized using solver added in Microsoft Excel 2016. The overall goodness fit for all measured and modelled inhibition zone was determined based on coefficient of determination (R^2) and the residual standard deviation (RSD) (Eq 3):

$$RSD = \sqrt{\frac{1}{n-c}\sum_{i=1}^{n}(M_i - P_i)^2} \qquad (3)$$

where Mi and Pi are the measured and predicted AgNPs/Ag$_{in}$, n is the number of the observed values and c is the number of fitted coefficients in equation 2 (typically 2).

RESULTS AND DISCUSSION

Effect of silver forms on the survival of soil microorganisms using disc diffusion assay

In the present study soil bacterial colonies on nutrient agar plates were used to investigate the antimicrobial properties of silver from different species (dissolved as Ag^+ and AgNPs) using disc diffusion assay. The results obtained from both silver forms show an increase of inhibition zone area with increasing Ag concentrations. The AgNPs showed high antimicrobial activity and exhibited larger zone area of inhibition. The AgNPs concentration of 2000 mgl^{-1}, showed highest inhibition zone compare to other concentration treatments. Figures (3 and 4) showed liner correlation between inhibition zone areas and different Ag and AgNPs concentrations. Significant correlation coefficients (Pearson coefficient; r) have been obtained (<0.05) between A_{in} and both Ag^+ (r = 0.96) and AgNPs (r = 0.87).
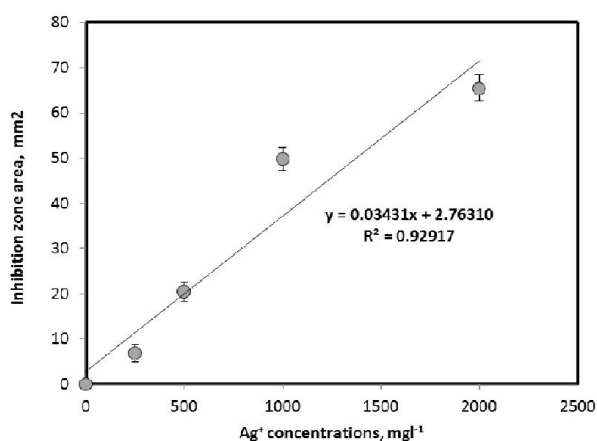

Figure (3): Effect of different Ag^+ concentrations on inhibition zone area for soil bacteria using disc difusion assay.
The solid line represents liner relationship. Error bars represent standered error of the three replicates.
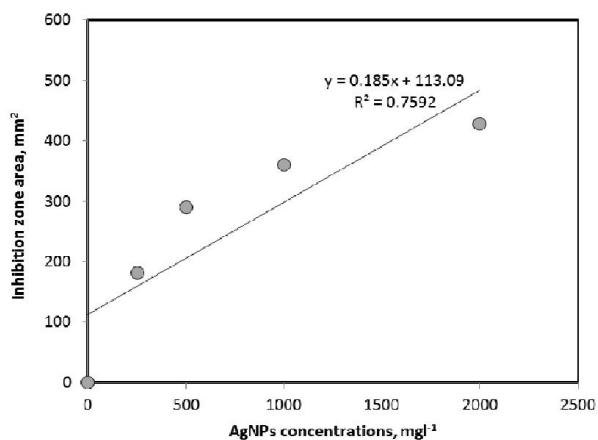

Figure (4): Effect of different AgNPs concentrations on inhibition zone area for soil bacteria using disc difusion assay.
The solid line represents liner relationship.
Error bars represent standered error (SE) of the three replicates (very samll SE values so it could not be seen).

The highest inhibition zone area was observed at the highest Ag concentrations (2000 mgl^{-1}); (mean ± standard deviation) 56.5 ± 2.89 and 428 ± 5.66 mm^2 for Ag^+ and AgNPs, respectively. However, the lowest inhibition zone area was observed with the lowest Ag concentrations (250 mgl^{-1}); 6.81 ± 1.83 and 181 ± 2.48 mm^2 for Ag^+ and AgNPs, respectively. The results showed that with the lowest AgNPs concentration the inhibition zone area showed 10 fold of magnitude compare to the lowest Ag^+ concentration. This finding confirms that the Ag nanoparticles are more antimicrobial active than that of Ag ions, by which the effect of antimicrobial is a nano-specific effects.

Our finding was in corresponding with Hänsch and Emmerling (2010). The authors added different concentrations of AgNPs (3.2 – 320 µg Ag kg^{-1}) to the soil directly and they found that soil microbial biomass decreased with increasing AgNPs concentrations and that it was significantly less than the control (p ≤ 0.05). A microcosm study using arctic soils suggested that AgNPs could significantly change the structure of soil microbial communities (Kumar *et al.*, 2014). Therefore, there is a suspect of AgNPs resistance by bacteria after long term of contact time between AgNPs and soil microbial community resulting in a new strain that can survive under AgNPs stress conditions.

The toxicity of AgNPs to soil bacteria has been investigated in more depth than has occurred for higher organisms (e.g. plants). However, toxicity testing has rarely been carried out using a natural soil; instead, pure culture solution assays are usually used. Fajardo *et al.* (2014) exposed pure cultures of two common soil bacteria, e.g. *Bacillus cereus* and *Pseudomonas stutzeri* to AgNPs. They reported significant toxicity for *B. cereus* and *P. stutzeri* following exposure to AgNPs at concentrations of 0.5 and 5 mg AgNPs l^{-1}, respectively. Toxic effects of AgNPs to rhizosphere bacteria have also been reported (Mirzajani *et al.*, 2013). Rhizosphere bacteria were isolated from a natural soil and cultivated in culture media and exposed to AgNPs. The minimum inhibition concentrations (MIC) of 7 isolated Bacillus bacteria were between 1 and 20 mg AgNPs l^{-1} (Mirzajani *et al.*, 2013). More complex methods for toxicity testing were used by Kumar *et al.* (2014) in their studies on the impact of AgNPs on arctic soil bacterial assemblages. Their methods included phospholipid fatty acid analysis, differential respiration, PCR-denaturing gradient gel electrophoresis and DNA sequencing. The authors found that soil microbial communities were affected by AgNPs when exposed to AgNPs at concentrations of 0.066% and 6.6% (w/w). They also demonstrated that Pseudomonas and Janthino bacterium bacteria were more resistant to AgNPs than bacterial species from other genera.

Predicting AgNPs/Ag$_{in}$ ratio

Attempts to predict AgNPs/Ag$_{in}$ ratio as a function of AgNPs concentrations. A power equation has been used as described in equation 2. Figure (5) shows the effect of different AgNPs concentrations on AgNPs/Ag$_{in}$ ratio. The model performance produced an acceptable result. These results have been justified depending on RSD (Eq 3) and R^2 values that showed a

significant correlation between measured and modelled values at p-values less than 0.01. Moreover, the RSD value which represent the estimated model error showed the lower value (1.31; Fig 5). The reason behind developing such model is to know which dissolved Ag concentration will equate to AgNPs concentration in terms of inhibition zone area.
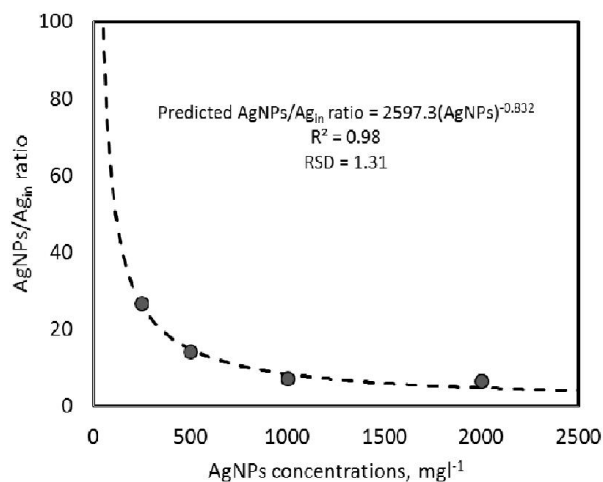

Figure (5): The ratio of inhibition zone area (AgNPs/Ag$_{in}$) as a function of different concentrations of AgNPs.
Broken line represents modelled values of AgNPs/Ag$_{in}$ ratio (Eq 2).

Therefore, from the unity value predicted from equation 2 (Figure 5), the unity ratio (AgNPs/Ag$_{in}$= 1) was obtained at the predicted AgNPs concentration of 12720 mgl^{-1}. The predicted inhibition zone area of silver nanoparticles (AgNPs$_{in}$) that corresponded to the concentrations of 12720 mgl^{-1} AgNPs is 2466 mm^2 according to the correlation equation in Figure (4) (AgNPs$_{in}$ = 0.185 (AgNPs) + 113.09). The estimated Ag$^+$ concentration is 71802 mgl^{-1} according to the correlation equation in Figure (3) (Ag$^+_{in}$ = 0.03431(Ag$^+$) + 2.76310). These estimated values gained from the developed model imply that the antimicrobial effects of AgNPs equal to approximately 5 times of dissolved Ag$^+$. Although a way that use a pure empirical model to predict AgNPs/Ag$_{in}$ ratio from AgNPs concentrations at high silver amount might need an experimental work to be validated, at worst it provides a relative assessment of AgNPs impact on microbial mortality comparing with Ag$^+$. Moreover, Figure (5) shows that predicted AgNPs/Ag$_{in}$ ratio (inhibition zone area ratio) increased with decreasing AgNPs concentrations. This finding indicate that the lower concentrations of AgNPs produced larger inhibition zone area comparing with the same concentration of dissolved Ag, again confirming the powerful of AgNPs as antimicrobial and as a nano-specific effect. In one of the few studies that have used a natural soil to investigate the effects of AgNPs on soil bacteria, the potency of AgNPs was reduced by soil components, *e.g.* clay (Calder *et al.*, 2012). Therefore, more soil needs to be used to study the effect of mitigating AgNPs toxicity as affected by different soil physio-chemical characteristics.

CONCLUSIONS

It is interesting to draw a clear conclusion from this work which confirms that Ag antimicrobial effect is a nano-specific. Moreover, the prediction model demonstrated that the effect of AgNPs is approximately 5 times higher than that of dissolved Ag. This work behaves as an alert precaution of using AgNPs without considering their ultimate disposal in soil. More work is needed to study the mechanism of toxicity effect of AgNPs on soil beneficial microbial community at short and long term applications. Moreover, different bacteria strains are also needed to be studied to see if AgNPs toxicity effect is also for a specific strain or not. However, as discussed above, soil physico-chemical properties might play an important role in determinant of AgNPs toxicity in soil microbial community. Therefore, the future works will focus on studying the effect of different soil components (*e.g.* Caly, organic matter, etc) on mitigating AgNPs toxicity in soil with specific microbial strains.

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تأثير جزيئات الفضة النانوية والذائبة على كائنات التربة الدقيقة

عزت رشاد مرزوق

قسم الأراضي و المياه ، كلية العلوم الزراعية البيئية ، جامعة العريش ، شمال سيناء ، رقم بريدي ٤٥٥١٦

تعتبر جزيئات الفضة النانوية من الجزيئات الأكثر استخداما على النطاق التجاري والتي تستخدم في العديد من المنتجات الاستهلاكية نظرا لتأثيرها المضاد للبكتريا. على الوجه الآخر فان تأثيرها المضاد للبكتريا يمكن أن يشكل أثر سلبي على مجتمعات ميكروبات التربة. في الدراسة الحالية تم تقييم ما إذا كان التأثير المضاد لميكروبات التربة راجع إلى الفضة في حجم النانو أم إلى الايونات الذائبة منها. تم دراسة تأثير استخدام جزيئات الفضة النانوية المصنعة بواسطة الكيمياء الخضراء (باستخدام مكونات طبيعية مثل النشا) و كأيونات فضة ذائبة (نترات الفضة) كمضادات لميكروبات التربة باستخدام طريقة أقراص الانتشار. تم دراسة نمو ميكروبات التربة من خلال قياس مساحة منطقة تثبيط النمو الميكروبي تحت تأثير تركيزات مختلفة من كلا من جزيئات الفضة النانوية والفضة الذائبة كنترات فضة (صفر – ٢٥٠ – ٥٠٠ – ١٠٠٠ – ٢٠٠٠ ملليجرام للتر). أظهرت الناتج أن تأثير جزيئات الفضة النانوية كمضادات لنمو ميكروبات التربة تعادل عشرة أضعاف تأثير الفضة الذائبة و ذلك عند التركيز المنخفض (٢٥٠ ملليجرام لتر⁻¹). إضافة إلى ذلك أوضحت نتائج التنبؤ بنسبة المساحة المثبطة نتيجة استخدام نسبة جزيئات الفضة النانوية إلى الفضة الذائبة إلى أن تأثير الفضة النانوية كمضادات لميكروبات التربة تعادل حوالي ٥ مرات تأثير الفضة الذائبة عندما تساوى النسبة المتنبأ بها الواحد الصحيح. على الرغم من أن الدراسة الحالية تأكد التأثير المثبط لميكروبات التربة بواسطة الفضة في الصورة النانوية إلا انه يجب استخدام العديد من الميكروبات المعزولة من العديد من الترب لدراسة تأثير الخواص الفيزوكيميائية لتلك الترب على تقليل الأثر السام لجزيئات الفضة النانوية على المجتمعات الميكروبية.