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Effect of Silver Nanoparticles on the Rhizosphere Exoenzyme of *T. orientalis Presl*

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Abstract: Silver nanoparticles（AgNPs）were extensively used in medicine as an antimicrobial agent. The unavoidable and extensive usage of AgNPs leads to accumulating in environment. Rhizosphere microenvironments are the essential base of terrestrial ecosystems and are ready to be disturb by environmental pollutants. In the present study, we investigated effects of AgNPs in rhizosphere soils by measuring activities of the exoenzymes urease, dehydrogenase, acid phosphatase, neutral phosphatase, and alkaline phosphatase. The effect of the concentrations of AgNPs (0.024, 0.24, 4.80, 9.60 μg/g dry soil) on the wetland plant *Typha orientalis Presl* was studied. In general, AgNPs were capable of inhibiting the activities of all the exoenzymes tested in this study. Especially, the urease, dehydrogenase and phosphatase activities in rhizosphere soil were significantly inhibited in high AgNP level (4.80, 9.60 μg/g dry soil); however, the low concentration of AgNPs (0.024 μg/g dry soil) only had the major adverse effect to exoenzyme activities of rhizosphere soil of *Typha orientalis Presl*. This study suggested that high concentration AgNPs negatively affect rhizosphere soil exoenzyme activities.
Keywords: silver nanoparticles (AgNPs); rhizosphere soil; soil exoenzymes activities; wetland plant; *Typha orientalis Presl*

1. Introduction

The rapid growth of the nanotechnology has resulted in the increasing production and use of engineered nanoparticles (ENPs) with the huge applications in several fields (www. nanotechproject.org). There is an estimated inventory of 1814 consumer products of ENPs identified in October 2013, with 435 products of silver nanoparticles (AgNPs) (Marina *et al.* 2015). Compared with other ENPs, AgNPs has huge applications due to its antimicrobial activity in several commercial and medical products, including wound dressing, textiles, water filters, algicides, disinfectants, deodorants, toothpaste, and house appliances (Yehia and Erik 2012,

[1), 2)] Ph.D

Brend *et al.* 2011, Thabet *et al.* 2010). As a consequence, the wide application of silver nanoproducts results in the release of AgNPs from them, with their inflow into the environment (Fadri and Brend 2011). AgNPs may adversely affect the activities of microorganisms within the environment (Christian *et al.* 2011).

In recent years the effect of AgNPs to soil is more and more popular as a research project due to its presence in soil. AgNPs would more likely accumulate in soil of a constructed wetland. Microorganisms is the basis of a constructed wetland, which determine the running status and quality of outlet. Studies on the impact of AgNPs on soil microorganisms are an important project. Most studies have concluded that the Ag^+ released from the nanoparticles is the main chemical species contributing to toxicity (Levard *et al.* 2013, Xiu *et al.* 2012), while several studies have found the effects of nanoparticles themselves to microorganisms like oxidative stress (Arugete and Hocella 2010, Fabrega *et al.* 2009), and direct interaction and uptake by bacteria (Morones *et al.* 2005). Furthermore, there have been relatively few studies on the impact of AgNP performed directly on the terrestrial environment, which is a chemically and physically complex system. Many studies focus on nanoparticles toxicity to bacteria (Liang *et al.* 2010, Yuan *et al.* 2013, Luo *et al.* 2014). AgNPs may well directly affect to soil microorganisms and indirectly affect to soil enzymes because of the high affinity of silver for soil organic matter and thiol compounds (Jacobson *et al.* 2005). As a result, AgNP contamination in soils could potentially affect the numerous biogeochemical processes, including nutrient cycling and a large majority of microbially mediated processes.

Enzymes found in soils have the biochemical and microbiological role and are “sensors” of soil health (Caldwell 2005). Urease, dehydrogenase and phosphatases are among the most frequently evaluated soil enzymes (Burns *et al.* 2013). Research in the effects of nanoparticles on soil enzyme is relatively few, but many researchs studied the effects of nanoparticles to pure soil were studied. Through long-term study, Du *et al.* (2011) found that TiO_2 and ZnO nanoparticles had affected the protease, catalase and peroxidase, but these nanoparticles had on effect on urease (Du *et al.* 2011). Shin et al evaluated the activities of 6 kinds of soil enzymes under stress of AgNPs, they found that AgNPs could restrain all enzyme activities, specially, could significantly inhibit the activities of dehydrogenase and urease (Shin *et al.* 2012). Raliya *et al.* (2015) explored the influence of TiO_2 nanoparticles on mung bean, they found that the activities of acid phosphatase, alkaline phosphatase and dehydrogenase with the rise of 67.3%, 72% and 108.7% respectively (Raliya *et al.* 2015). Soil enzyme assays have demonstrated potential for the early detection of anthropogenic or natural disturbances as well as a proven sensitivity for evaluating the effects of trace metals in contaminated soils (Chaperon and Sauve 2007, Zhang *et al.* 2010).

This study focused on evaluating the short-term effect of AgNPs on the activities of common exoenzymes found in rhizosphere soils of *Typha orientalis Presl*. The changes in the activities of exoenzymes related to removing abilities of constructed wetland to nitrogen and phosphorus, are measured using urease acid phosphatase, neutral phosphatase and alkaline phosphatase, respectively. The overall microbial activity is predicted by assessing the dehydrogenase activities. Thus, the study attempts to explore the influences of AgNP on exoenzymes in

rhizosphere soil of the wetland plant *Typha orientalis Presl*, indirectly reflecting the degree of AgNP stress on rhizosphere. It also lays the foundation wetlands to understand the changes of decontamination ability under AgNP stress.

2. Materials and methods

2.1 Mesocosms

The mesocosms were circular shaped plastic drum and were kept indoors in the city of Nanjing, Jiangsu Province. Barrel mouth diameter and internal diameter at bottom of each mesocosm was 30 cm, 20 cm, respectively. Each mesocosm was 31 cm deep. At the bottom of mesocosms was covered with 5 cm thick gravel. 6 kilograms soils were added to provide a uniform 15 cm layer of soil over the gravel layer in the mesocosm. The soil used in the experiment had an average content of 17.02% moisture. These loam soils were taken from Siming mountain of Nanjing.

The species of wetland plant *Typha orientalis Presl* used in the study were purchased from a weland at the Muyang county, Jiangsu province, China on the November 2013. These plants were cultured in the same nutrient solution under common external environment. And then healthy, same growing plants were chosen as the supplied experimental plants. These selected plants were planted in the mesocosms on the January 2014, about 3 months prior to dosing with AgNPs.

2.2 AgNP stock suspensions

The Ag nanoparticles used in this study were purchased as monomer suspensions (Huzheng nano technology co., LTD, Shanghai, China). They were reported by the manufacturer to be roughly spherical and 15 nm in diameter with a PVP coating (2000 mg/L). The particles were polydisperse with particle sizes ranging from 10 to 40 nm in diameter based on TEM (JEM-2100, JEOL LTD.) and DLS measurements (z-average hydrodynamic diameter).

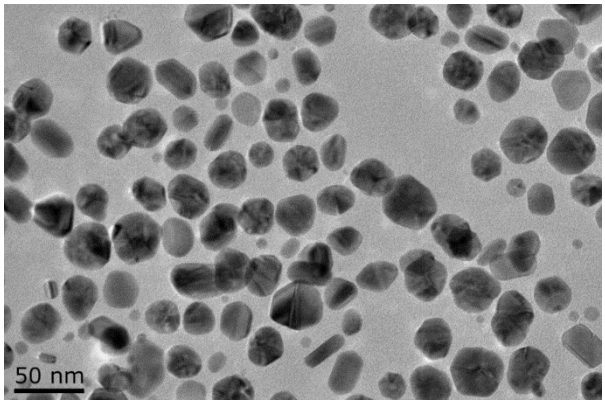

Fig. 1. TEM photograph of nanometer silver solution

2.3 Dosing AgNPs

AgNPs were dosed into the mesocosms on the April 1, 2014. During the test the ambient temperature was about 20-29℃ in day and about 12-22℃ at night. The experiment was

originally a randomized block design and five nanoparticle treatments: 0, 0.024, 0.24, 4.80, 9.60 μg/g dry soil, with 3 replicates per treatment. AgNP dispersions were prepared at 40 mg/L by diluting 0.4L of 2000 mg/L PVP-AgNPs to 20 L AgNPs suspensions. A total of 45 batches were prepared and combined to provide to a total of 64.8 L of suspension for dosing the mesocosms. Finally, there is 1.44 L suspension per replicate which was being added into the 6 kg of soil of mesocosms and its concentration was 40.0 mg/L, 20.0 mg/L, 1.00 mg/L, 0.100 mg/L, 0.00 mg/L respectively. Each concentration of AgNPs dispersions had 12.96 liters and the suspensions were placed in a plastic vessel, pressurized, and then sprayed evenly over the soil, respectively. Care was taken to minimize the amount of solution that laned on plant leaves and stems. The concentration of Ag in soil was tested by ICP-MS (7700, Agilent company) after dosing the AgNP suspensions. The results are as Table 1.

Table 1. the concentration of AgNPs in mesocosms (unit: μg/g dry soil)

	AgNPs	AgNPs	AgNPs	AgNPs	Deionized water
Target concentration	0.0240	0.240	4.80	9.60	—
Actual concentration	0.0269	0.269	5.35	10.4	—

2.4 Assay of enzyme activities

After dosing, four times of the enzyme assays were performed: April 7, April 14, April 21, April 28, 2014, and following the methods appeared in the publications by Tabatabai 1994, Gong 1997, Kandeler and Gerber 1988, An and Kim 2009. All the enzyme activities were measured based on colorimetric determinations using a UV/Vis spectrophotometer (UV1800, Jinghua Technology Instrument Co., LTD, Shanghai, China). Soil (dehydrogenase: 2.5g, other 4enzymes: 1.0g) was weighted into a glass test tube with stopper and suspended in buffered substrate solutions. The samples were capped, thoroughly mixted on a vortex shaker, and placed in a constant temperature incubator in the dark. TRIS buffer (1M, pH 8.0), borate buffer (0.05M, pH 10.0), acetate buffer (pH 5.0), borate buffer (pH 9.4), and citrate buffer (pH 7.0) were used as the buffer solutions for the dehydrogenase, urease, acid phosphatase, neutral phosphatase and alkaline phosphatase assays, respectivrly. INT (0.2 mM), urea (0.02M) and PNP (0.05M) were used as substrates for dehydrogenase, urease and phosphatase assays, respectively. The incubation periods for dehydrogenase and urease were 24h, 2h, respectively, and those of acid, neutral and alkaline phosphatase were 24h.

2.5 Statistical analysis

The IC50 value, which is the AgNP concentration causing 50% inhibition of the enzyme activity compared to the control; the IC30 value, which is the AgNPs concentration causing 30% inhibition of the enzyme activity compared to the control, was calculated by expressing each treatment as a proportion of the control using the Trimmed Spearman-Karber method (Hamilton *et al.* 1977). Differences between treatments for the different measured variables were tested

using one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test when significant differences were found ($p < 0.05$).

3. Results and Discussions

3.1 Effect of AgNPs on activities of dehydrogenase

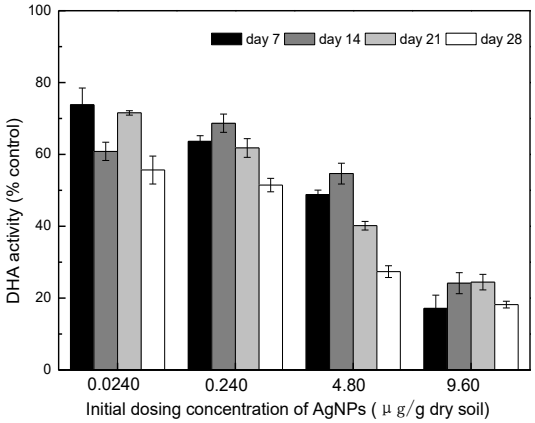

Fig. 2. Changes in the rate of dehydrogenase (DHA) activity under AgNP stress

(The change rates shown in the figure refer to the percentages of the control (no AgNP) exposed to 0.0240, 0.240, 4.80, 9.60 μg/g dry soil on days 7, 14, 21, 28 in rhizosphere soil of *Typha orientalis Presl.* Values are the means of three replicates ± standard deviation.)

$$XH2+A \xrightarrow{\text{dehydrogenase}} X+AH2 \qquad (3\text{-}1)$$

As an important enzyme involved in the conversion of soil organic matter, dehydrogenase can promote dehydrogenation reaction of carbohydrates, organic compounds, playing the important role of intermediate carrier of hydrogen, such as the formula (3-1) below. Fig. 2 shows that, inhibition degree of dehydrogenase was more significant under high AgNP concentrations (0.240,4.80,9.60 μg/g dry soil), and also the degree of inhibition increased with the increase of the concentration of AgNPs. On the one hand, high concentrations of AgNPs was toxic on rhizosphere microbes (Navarro *et al.* 2008, Choi *et al.* 2008, Das *et al.* 2012, Garcia *et al.* 2012), leading to decrease of the quantity of soil microorganisms itself and the capacity of enzyme production (Hemida *et al.* 1997). On the other hand, higher AgNP concentration also reduced the plant root activity and affected the generation of root exudates, which could influence activity of dehydrogenase. While under 0.0240 μg/g dry soil of AgNPs, for the dehydrogenase from rhizosphere of *Typha orientalis Presl*, there had a significant inhibition. Root exudates of *Typha orientalis Presl* would let the activity of dehydrogenase generate different changes at low AgNP concentration, because the root exudates can affect the abundance and community structure of rhizosphere microorganisms, directly or indirectly affecting the dehydrogenase activity. Since dehydrogenase is an important indicator to reflect microbial activities, organic degradation and root absorption capacity, the activity of dehydrogenase was supressed in this study, indicating a certain degree of inhibition to the activity of soil microorganism, degradation and root absorption of organic compounds under AgNP stress.

3.2 Effect of AgNPs on activities of urease

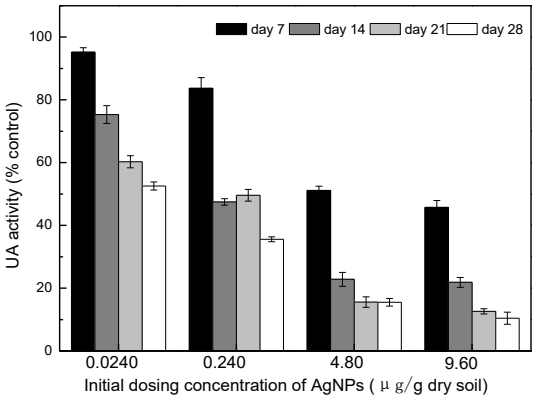

Fig. 3. Changes in the rate of urease (UA) activity under AgNP stress

(The change rates shown in the figure refer to the percentages of the control (no AgNP) exposed to 0.0240, 0.240, 4.80, 9.60 μg/g dry soil on days 7, 14, 21, 28 in rhizosphere soil of *Typha orientalis Presl.* Values are the means of three replicates ± standard deviation.)

The Fig.3 shows that, the inhibition degree of urease activity was more obvious on high AgNP concentrations (4.80, 9.60 μg/g dry soil). Partly reason is considered that urease is highly sensitive to various metals, especially for Ag (or Ag^{+}). Inhibition mechanism of high AgNPs concentrations on urease activity may be due to AgNP itself or dissolved silver ions, as noncompetitive inhibitor, which could bind with important catalytic thiol group (-SH) of urease molecule, thereby inhibiting the urease activity. On the other hand, AgNPs could affect microorganisms, root exudates and animals associated with urease, thus inhibiting the production of urease. At low AgNP concentrations (0.024, 0.240 μg/g dry soil), urease activty in rhizosphere soil of *Typha orientalis Presl* was been obvious inhibited. Since microbial quantity, organic matter content of the soil matrix and total nitrogen and available nitrogen content was positively correlated with urease activity. Thus, the activity of urease suppressed, indicating the decrease of microbial population in rhizosphere soil under AgNP stress, and nitrogen cycle of the rhizosphere to some extent repression.

3.3 Effect of AgNPs on activities of phosphatase

Phosphatase is an important enzyme for biological phosphorus metabolism, catalyzing hydrolysis of phosphate monoester and releasing inorganic phosphate (Rogers and Reithel 1960). The activity of phosphatase is the indicatorst to evaluate direction and strength of phosphorus biotransformation. Depending on the optimum pH, phosphatase can divide into acidic phosphatase, alkaline neutral phosphatase. Acid phosphatase is widely present in plants and soil, and plays a highly important role in transformation of phosphorus in soil and aquatic systems (Pierzynski *et al.* 2005). The activity of acid phosphatase can respect content of phosphorus to a certain extent. Alkaline phosphate can converte to active bioavailable phosphorus, and plays an important role in the mineralization of organic phosphorus (Chrost *et*

al. 1984).

3.3.1 Effect of AgNPs on activities of acid phosphatase

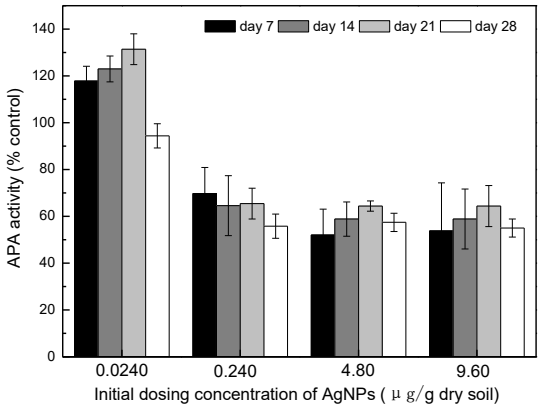

Fig. 4. Changes in the rate of acid phosphatase (APA) activity under AgNP stress

(The change rates shown in the figure refer to the percentages of the control (no AgNP) exposed to 0.0240, 0.240, 4.80, 9.60 μg/g dry soil on days 7, 14, 21, 28 in rhizosphere soil of *Typha orientalis Presl.* Values are the means of three replicates ± standard deviation.)

Compared with the dehydrogenase and urease, Fig. 4 shows that, the degree of inhibition of acid phosphatase was relatively low. Under stress of high AgNP concentrations (4.80, 9.60 μg/g dry soil), the level of inhibition to activity of acid phosphatase in the rhizosphere soil was relatively significant, because the high concentrations of AgNPs in rhizosphere soil affects microorganisms associated with acid phosphatase, root exudates, animals (Unrine *et al.* 2012). The acid phosphatase from rhizosphere soil of *Typha orientalis Presl* had significant inhibition at 0.240 μg/g dry soil AgNPs, while under stress of AgNP at 0.0240 μg/g dry soil there was no significant inhibition on acid phosphatase.

3.3.2 Effect of AgNPs on activities of neutral phosphatase

Inhibition to activity of neutral phosphatase in rhizosphere soil was relatively low compared with the dehydrogenase and urease in Fig. 5. Activity of neutral phosphatase of *Typha orientalis Presl* under AgNP stress of high concentrations (4.80, 9.60 μg/g dry soil) was slightly inhibited. The difference is that behave of neutral phosphatase in rhizosphere soil of *Typha orientalis Presl* at low AgNP concentration (0.0240, 0.240 μg/g dry soil) was that, the activity increased in the initial stage, however the latter the activity declined, and the degree of inhibition was similar with high concentrations.

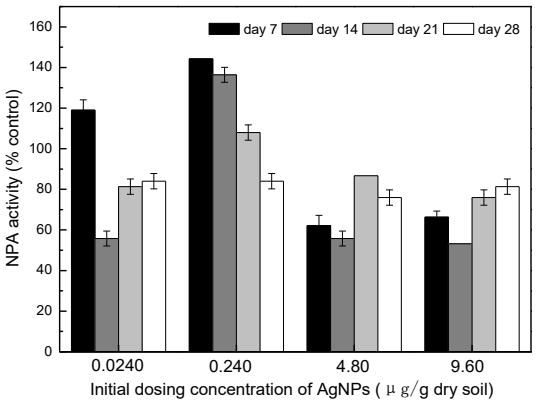

Fig. 5. Changes in the rate of neutral phosphatase (NPA) activity under AgNP stress

(The change rates shown in the figure refer to the percentages of the control (no AgNP) exposed to 0.0240, 0.240, 4.80, 9.60 μg/g dry soil on days 7, 14, 21, 28 in rhizosphere soil of *Typha orientalis Presl.* Values are the means of three replicates ± standard deviation.)

Since the background value of neutral phosphatase activity in the test soil was low (only 1/3 activities of acid, alkaline phosphatase), slight changes of its activity can lead to great volatility of the change rate of neutral phosphatase. It suggested that neutral phosphatase activity at low AgNP concentration was not significantly affected, a slight fluctuation within the normal range. Under AgNP stress of high concentrations, the neutral phosphatase activity decreased, indicating that high AgNPs concentration had an impact on rhizosphere microorganisms, root exudates, animals associated with neutral phosphatase, thus inhibiting the generation of neutral phosphatase (Roberts *et al.* 2007, Adams *et al.* 2006).

3.3.3 Effect of AgNPs on activities of alkaline phosphatase

The activity of alkaline phosphatase in rhizosphere soil of *Typha orientalis Presl* had obvious decline at high AgNP concentrations (0.240, 4.80, 9.60 μg/g dry soil). Thus, under stress of high AgNP concentrations (4.80, 9.60 μg/g dry soil), inhibiting degree of acid phosphatase in rhizosphere soil of all kinds of wetland plants was relatively significant, because of the high concentration of AgNPs on rhizosphere soil that affected microorganisms, root exudates and animals associated with acid phosphatase [오류! 책갈피가 정의되어 있지 않습니다.,오류! 책갈피가 정의되어 있지 않습니다.]. Similarly, differences in the root exudates of different species caused alkaline phosphatase showed different changes at low inhibitory effect of AgNP.

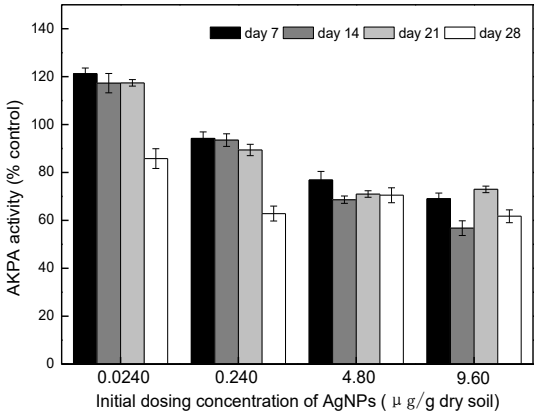

Fig. 6. Changes in the rate of alkaline phosphatase (AKPA) activity under AgNP stress

(The change rates shown in the figure refer to the percentages of the control (no AgNP) exposed to 0.0240, 0.240, 4.80, 9.60 μg/g dry soil on days 7, 14, 21, 28 in rhizosphere soil of *Typha orientalis Presl.* Values are the means of three replicates ± standard deviation.)

4 The inhibition concentrations for enzyme activities exposed to AgNPs

Table 2.The inhibition concentrations (IC50) for the exoenzyme activities exposed to AgNPs in different rhizosphere soil after day 28 (unit: μg/g dry soil)

Species	Typha orientalis Presl	
enzymes	IC50[a]	IC30[a]
Dehydrogenase	0.09504（0.0216-0.2422）	-
Urease	0.03672（0.02064-0.05832）	-
Acid phosphatase	>9.60	0.448（0.0228-1.991）
Neutral phosphatase	NC[b]	NC[b]
Alkaline phosphatase	>9.60	0.912（0.0244-22.077）

[a] Timmed Spearman-Karber method. Values in parentheses represent the 95% confidence level.

[b] Not calculable.

Using the software SPSS19.0 (International Business Machines Corporation, Aromnk, New York, America) to calculate the inhibition concentrations for soil enzyme activities under AgNP stress, the results are shown in Table 2. From the table, the values of IC50 for dehydroganase and urease are less than 4.80 μg/g dry soil, indicating that the stress of AgNP at 4.80, 9.60 μg/g dry soil lets the degree of inhibition of the activities of dehydrogenase and urease to 50% at the end of the experiment (28 days). However, the IC50 value for dehydrogenase was just 0.09504 μg/g dry soil, whih was greater than the concentration at 0.240 μg/g dry soil of AgNPs, revealing that it had 50% dehydrogenase been inhibited under the AgNP stress of 0.240 μg/g dry soil.

It is shown that after 28 days, IC50 value of acid and alkaline phosphatase was higher than

9.60 μg/g dry soil, showing the extent of inhibition did not reach 50% within the scope of the test. Value of IC30 of acid and alkaline phosphatase was less than 4.80 μg/g dry soil. The activity of netural phosphatase had poor regularity with the changes of concentrations of AgNPs, wherefore it cans not calculate the values of the inhibition concentrations of netural phosphatase.

5 Conclusions

A numerical simulation procedure for predicting directional typhoon wind fields over complex terrain has been proposed in this study.

·Under AgNP stress from high concentrations (4.80, 9.60 μg/g dry soil), the degree of inhibition to dehydrogenase and urease was significant, and rised with increase of concentrations of AgNPs.

·Compared to the dehydrogenase and urease, the stress of AgNP on phosphatase in rhizosphere was lower, but there was a significant inhibition at high concentrations of AgNPs.

·Activities of dehydrogenase, urease and phosphatase suffered significantly restrain, showing there was some influences on rhizosphere microorganisms, absorption of organic matter, nitrogen and phosphorus cycles.

Acknowledgements

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