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Inhibition Kinetics of Ammonia Oxidation Influenced by Silver Nanoparticles

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Abstract Silver nanoparticles (AgNPs) have significantly increased in production and use for anti-microbial propose. This agent, after used, is released into sewerage system resulting in possibility to inactivate non-targeted microorganisms in wastewater treatment plants. In this study, the inhibitory effect of AgNPs on ammonia oxidation was investigated using respirometric assay. The initial concentrations of AgNPs and ammonia ranged 0.25–10.00 and 14–280 mg/L, respectively. Half saturation constant (K_s) for ammonia oxidation was found to be 15.9 mg N/L. Under the presence of AgNPs, the maximum oxygen uptake rate and K_s declined. The effect of AgNPs was proved to follow an uncompetitive-like inhibition kinetic type with the inhibition coefficients (K_i) of 5.5 mg/L. Increasing AgNPs from 0.25 to 10.00 mg/L inhibited 4 to 50 % of ammonia-oxidizing activities at the initial ammonia concentrations from 14 to 280 mg/L. Based on transmission electron microscopic observation, AgNPs could damage the microbial cells. All findings indicated that AgNPs substantially reduced ammonia-oxidizing microorganisms and their activities. Thus, special attention should be made to manage discharge of AgNPs into the environment.

Keywords Ammonia oxidation · Inhibitory kinetics · Nitrifying activated sludge · Respirometer · Silver nanoparticles

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1 Introduction

Silver nanoparticles (AgNPs) have been widely used as an anti-microbial agent in a variety of goods, such as personal care products, home commodities, and food services. It was previously predicted that biocidal products containing AgNPs would reach 110–230 tons/year by 2010 in Europe (Blaser et al. 2008; Sharma et al. 2009; Kahru and Dubourguier 2009). Silver nanoparticles have gained public attention because they were proved on their negative impact to several living organisms. Silver nanoparticles can be

toxic to cells by inhibiting gene expression, damaging chromosome, and creating oxidative stress in the cells (Lu et al. 2008; Kim et al. 2009). In microorganisms, AgNPs cause DNA replication fidelity and form the pits attributing to cell disability or death (Sondi and Salopek-Sondi 2004). After widespread usage of AgNPs, they could be gathered and conveyed to wastewater treatment plants. In the plants, activity of AgNPs may still remain and thus adversely affect microorganisms resulting in failure of wastewater treatment plants.

Between heterotrophic and autotrophic microorganisms in wastewater treatment system, growth of autotrophs is known to be much slower, resulting in the difficulty to maintain such microorganisms in the systems. These microorganisms govern nitrification process which is the autotrophic oxidation of ammonia to nitrate. Nitrification comprises two-step processes by that ammonia is prior oxidized to nitrite by ammonia-oxidizing microorganisms (AOM), and nitrite is subsequently oxidized to nitrate by nitrite-oxidizing microorganisms (NOM). Comparing the two processes, ammonia oxidation is believed to be the rate-limiting step since AOM have slower growth rate and are more sensitive to environmental stress than NOM. Therefore, in this study, the oxidation of ammonia to nitrite was concerned.

Effects of toxic substances to ammonia oxidation in wastewater treatment plants have been intensively investigated. Several heavy metals, such as Cr^{3+}, Cr^{6+}, and Cd^{2+}, have been studied and postulated to cause significant effects on complete nitrification or ammonia oxidation in activated sludge systems (Battistoni et al. 1993; Cokgor et al. 2007). For AgNPs, there were only few previous studies relating these nanoparticles and complete nitrification (Choi et al. 2008; Choi and Hu 2008). However, all prior studies focused only on the impact of AgNP on complete nitrification efficiency at certain concentrations of AgNPs. Respiration rate of nitrifying bacteria was found to be inhibited by up to 41 % (Hu et al. 2010) and 86 % (Choi et al. 2008) at the AgNP concentration of 1 mg/L. To the best of our knowledge, the inhibitory kinetic information of ammonia oxidation including inhibition type, inhibitory kinetic constant, and ammonia oxidation performance has never been published. This work is the first report on kinetics of ammonia oxidation influenced by AgNPs. Transmission electron microscopic (TEM) observation of AgNP-affected cells was also performed for insight information.

2 Materials and Methods

2.1 Silver Nanoparticles

Obtained AgNPs were synthesized from silver nitrate by borohydride reduction methods which are described elsewhere (Ngeontaea et al. 2009). The spherical AgNPs with an average size of 14 nm were characterized using transmission electron microscopy. The size and shape were chosen because they are typically used in commercial products.

2.2 Enrichment Medium

The composition of enrichment medium was previously described (Limpiyakorn et al. 2007). A synthetic stock medium contained 0.33 g/L $(NH_4)_2SO_4$, 4.04 g/L Na_2HPO_4, 2.1 g/L K_2HPO_4, and 0.75 g/L $NaHCO_3$ with an addition of inorganic salt solution (1 mL). The inorganic salt solution comprised 40 g/L $MgSO_4\ 7H_2O$, 40 g/L $CaCl_2\ 2H_2O$, 200 g/L KH_2PO_4, 1 g/L $FeSO_4\ 7H_2O$, 0.1 g/L Na_2MoO_4, 0.2 g/L $MnCl_2\ 4H_2O$, 0.02 g/L $CuSO_4\ 5H_2O$, 0.1 g/L $ZnSO_4\ 7H_2O$, and 0.002 g/L $CoCl_2\ 6H_2O$. All chemicals were purchased from Merck Company (Darmstadt, Germany).

2.3 Enrichment of Nitrifying Activated Sludge

Seed sludge was collected from a municipal wastewater treatment plant and enriched in a 12-L sequencing batch reactor for 6 months. The hydraulic retention time and solid retention time of the reactor were 2 and 24 days, respectively. Temperature, pH, and dissolved oxygen (DO) concentration were maintained at 25–30 °C, 7–8, and above 2 mg/L, respectively.

2.4 Respirometric Experiments

The NAS was centrifuged and washed five times with an inorganic salt solution before being used in respirometric experiments. A respirometer was a 250-mL completely sealed glass vessel equipped with a DO probe (WTW GmbH, Weiheim, Germany). The washed NAS, with mixed liquor volatile suspended solid concentrations of 113±14 mg/L, was added into the vessel and filled up with the enrichment medium described above. The medium with pH range from 7.8 to 8.2 was prepared by phosphate buffer. Sodium azide (NaN_3) at a final concentration in the vessel of 24 μM

was added to inhibit the activity of NOM. The vessel was magnetically stirred and maintained at room temperature (23–25 °C). The initial AgNP concentrations varied at 0.00, 0.25, 0.50, 5.00, and 10.00 mg/L. With each concentration of AgNPs, four duplicate sets of respirometers were carried out with initial ammonia concentrations ranging from 14–280 mg N/L. All tests were run for 1.5 h. The DO values were continually monitored and recorded via the software from WTW GmbH (Weiheim, Germany). The DO values were used for oxygen uptake rate (OUR) calculation.

2.5 Calculation of Kinetic Parameters

Oxygen uptake rate was determined based on the change of DO concentrations over time Eq. (1).

$$\mathrm{OUR} = \frac{d[\mathrm{DO}]}{dt} \tag{1}$$

where [DO] is the DO concentration (in milligrams per liter) while t is time (in minutes).

$\mathrm{OUR}_{\max}$ and K_s values were computed from Michaelis–Menten in Eq. (2):

$$\mathrm{OUR} = \mathrm{OUR}_{\max}\frac{s}{K_s + s} \tag{2}$$

where OUR is the oxygen uptake rate (in milligrams of O_2 per liter per minute); $\mathrm{OUR}_{\max}$ is the maximum oxygen uptake rate (in milligrams of O_2 per liter per minute), s is the ammonia concentration (in milligrams of N per liter), K_s is the half saturation coefficient for ammonia (in milligrams of N per liter). Under the presence of inhibitor (AgNPs) Eq. (2) can be modified (Eq. 3):

$$\mathrm{OUR} = \mathrm{OUR}_{\max}\frac{s}{K_s + s\left(1 + \frac{I}{K_i}\right)} \tag{3}$$

where I is the concentration of AgNPs (in milligrams per liter), K_i is the inhibition constant (in milligrams per liter). Equation (3) can be taken as the reciprocal on both sides and become Lineweaver–Burk model (Eq. 4):

$$\frac{1}{\mathrm{OUR}} = \frac{K_s}{\mathrm{OUR}_{\max}}\frac{1}{s} + \frac{1}{\mathrm{OUR}_{\max}}\left(1 + \frac{I}{K_i}\right) \tag{4}$$

The K_i value can be estimated by plotting $K_s/\mathrm{OUR}_{\max}$ versus I. The intercept on the x-axis gives the $-K_i$ value. Additionally, the quantification of percentage of inhibition caused by AgNPs was calculated by comparing OUR of AgNPs experiments with the control as the following (Eq. 5):

$$\mathrm{Inhibition}\cdot(\%) = \frac{\left(\mathrm{OUR}_{\mathrm{control}} - \mathrm{OUR}_{\mathrm{AgNPs}}\right)\times 100}{\mathrm{OUR}_{\mathrm{control}}} \tag{5}$$

2.6 TEM Analytical Method

The NAS samples from selected experiments were chosen based on the ammonia-oxidizing activitiy. The NAS samples before and after the respirometric experiments were compared. The NAS samples were fixed by a glutaraldehyde solution of 2.5 % overnight at 4 °C. The fixed cells were washed by a series of solutions including the phosphate buffer and deionized water. The washed cells were mixed with an osmium tetroxide solution of 1 %. After that, the mixture was mixed with melt agar of 1.5 % to form the gel. The hardened agar was cut as a cube and was then dehydrated with 35, 50, 70, and 95 % of ethanol, and absolute ethanol, respectively.

The dehydrated cubic cells were saturated in propylene oxide and were then soaked with spur resin and propylene oxide mixture. Next, the cubes were baked at 70 °C for 8–10 h. The baked cubes were cut by an ultramicrotome. The cut cubes with sizes between 60 and 90 nm were pasted into copper grid and stained with uranyl acetate and lead citrate for increasing the contrast. The stained cubes with cells were observed using TEM (JEOL, JEM-2100, Tokyo, Japan).

3 Results and Discussions

3.1 Oxygen Consumption by Heterotrophs and Abiotic Reaction

Besides nitrifying microorganisms in NAS, there are possibilities that DO in the respirometeric experiments could be consumed by heterotrophs or abiotic reaction. To monitor these side reactions, two control experiments were conducted. For the heterotrophic activity, a control experiment in the medium containing NAS (no ammonia) was carried out. Another control experiment in the medium containing ammonia and AgNPs (no NAS) was performed to examine DO sink by abiotic reaction. Both control experiments

showed no significant depletion of DO concentrations. The OUR of 0.002 mg O_2/L/min was found for oxygen consumption experiment by heterotrophs, and the OUR of 0.001 mg O_2/L/min was found in the test for abiotic reaction. These results suggested that there was no significant interference by both heterotrophic and abiotic influences in this study.

3.2 Ammonia Oxidation Kinetics

Ammonia oxidation experiment was conducted at different ammonia concentrations ranging from 14 to 280 mg N/L. Figure 1 presents the result of OUR values for ammonia oxidation. The result from the tests without AgNPs well fitted to Michaelis–Menten model. The OUR_{max} of 0.2273 mg O_2/L/min and the K_s of 15.87 mg N/L were estimated (Table 1). The results are similar to the findings of Carrera et al. (2004) that the K_s values of 11 mg N/L for ammonia oxidation was investigated.

Generally, inhibition kinetics is based on enzyme reactions which are categorized to three kinds of inhibition mechanisms including competitive, non-competitive, and uncompetitive inhibitions. It was found that the experimental results from this study followed uncompetitive inhibition kinetics. Under the presence of AgNPs, an increase in AgNP concentrations resulted in decreasing OUR_{max} and K_s (Fig. 1 and Table 1). For the calculation of inhibitory

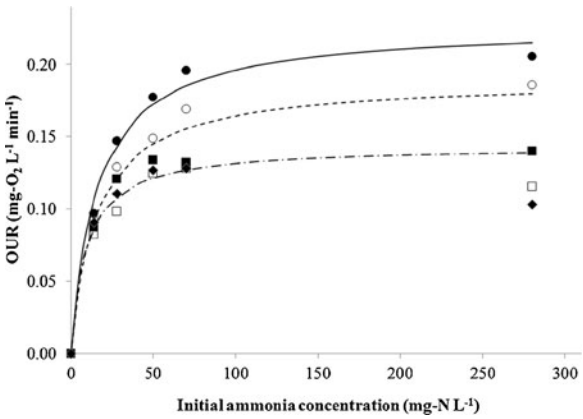

Fig. 1 Oxygen uptake rates for ammonia oxidation under the presence of AgNPs at concentrations of 0.00 (*white circle*), 0.25 (*black circle*), 0.50 (*black square*), 5.00 (*white square*), and 10 mg /L (*diamond*). Three lines (*line*, *dashed line*, and *dashed-dotted line*) present the uncompetitive inhibition curve fit for the tests with AgNPs at concentrations of 0.00, 0.25, and 0.50 mg/L, respectively

Table 1 OUR_{max} and K_s for ammonia oxidation under various AgNP concentrations

AgNPs (mg/L)	OUR_{max} (mg O_2/L/min)	K_s (mg N/L)	R^2
0.00	0.2273	15.87	0.989
0.25	0.1984	16.15	0.988
0.50	0.1564	9.99	0.994
5.00	0.1497	8.82	0.998
10.00	0.1448	8.33	0.999

coefficient, K_i, the experimental data of oxygen uptake rates and initial AgNP concentrations were fitted in the enzyme kinetic module. The calculated K_i value for ammonia oxidation was 5.5 mg/L.

Normally, it is known that metals, including silver, influence to cellular physiology, especially interactions with proteins (Ren and Frymier 2003). The proteins could be microbial organs, enzymes, or other materials such as nucleic acids. Therefore, AgNPs may play a role in enzyme catalytic reactions related to ammonia oxidation or cells were physically damaged. Silver nanoparticles exhibited more toxic than other nanoparticles (Ren and Frymier 2003). This study reported the K_i values from the effect of AgNPs on ammonia oxidation for the first time. Nevertheless, the K_i values obtained from this study may not be well compared to those of other heavy metals due to different experimental configurations (Ren and Frymier 2003). The calculated K_i values were used to indicate the toxicity of substances to microorganism. The lower the K_i means the higher the toxicity to target organisms (Eq. 2). The kinetic parameters could be used for further studies or practices.

3.3 Inhibition Estimation

Quantification of the inhibition of AgNPs for ammonia oxidation was also made in the present study (Table 2). Increasing AgNP or ammonia concentrations resulted in higher inhibition level. Silver nanoparticles and ammonia at the concentrations of 0.25–10.00 and 14–280 mg N/L, respectively, caused an inhibition of 4–50 % (Table 2).

The impact of common heavy metals in activated sludge has been investigated. It was reported that Cr^{6+} and Ni^{2+} inhibited microbial oxygen uptake rates up to 15 and 40 %, respectively, within 30-min tests (Cokgor et al. 2007). Respiration rates of nitrifying bacteria was

Table 2 Percent inhibition for ammonia oxidation under various AgNP concentrations

AgNP concentration (mg/L)	Inhibition (%)						
	Ammonia concentration (mg-N/L)						
	14	28	50	70	280	Average	
0.25	4	12	20	14	10	12	Low inhibition
0.50	9	18	22	33	32	23	↓
5.00	15	24	25	35	44	28	High inhibition
10.00	6	25	27	35	50	28	
Average	8	20	23	29	34		
	Low inhibition → High inhibition						

found to be inhibited by up to 41 % (Hu et al. 2010) and 86 % (Choi and Hu 2008) at the AgNP concentration of 1 mg/L. Hu et al. (2010) found that at concentrations of 0.40 and 0.75 mg/L, total Ag inhibited the growth of nitrifying bacteria by 11.5 and 50.0 %, respectively. The previous studies only focused on the inhibition of complete nitrification. However, based on nitrification stoichiometry, oxygen uptake in nitrification mainly comes from ammonia oxidation (75 % of total oxygen uptake). This could say that ammonia-oxidizing activity may be estimated as three-fourths of complete nitrifying activity. Among the literatures and the result from this study, it obviously indicates that AgNPs affect ammonia oxidation (or nitrification) differently. The discrepancy of AgNP effects might come from differences of cell concentration, characteristics of AgNPs, agglomeration of AgNPs, composition of communities as well as exposure time in the activated sludge.

Two inhibition mechanisms have been proposed for the effect of AgNPs on bacteria including formation of free radicals from the surface of AgNPs and damaging of microbial cell surface (Choi and Hu 2008; Panacek et al. 2009). Uncontrolled generation of the free radicals could attach on membrane lipids and then lead to a breakdown of membrane function (Choi et al. 2008). This may not be the reason for this study. Because by nature, free radicals consumed oxygen in the experimental environment; however, the control experiment was conducted and proved that there was no dissolved oxygen consumption. Moreover, it was reported that the free radicals generated by AgNPs took place only in the presence of UV light (Choi and Hu 2008). It is indicated that in this study, the inhibition of ammonia oxidation is not caused by the free radicals. Another suggested mechanism was the formation of "pits" in the cell wall of bacteria under the presence of AgNPs (Sondi and Salopek-Sondi 2004; Choi et al. 2008). Furthermore, it was recommended that the nanoparticles preferably attacked the respiratory system, cell division finally leading to cell death (Sondi and Salopek-Sondi 2004). This could be the reason of the

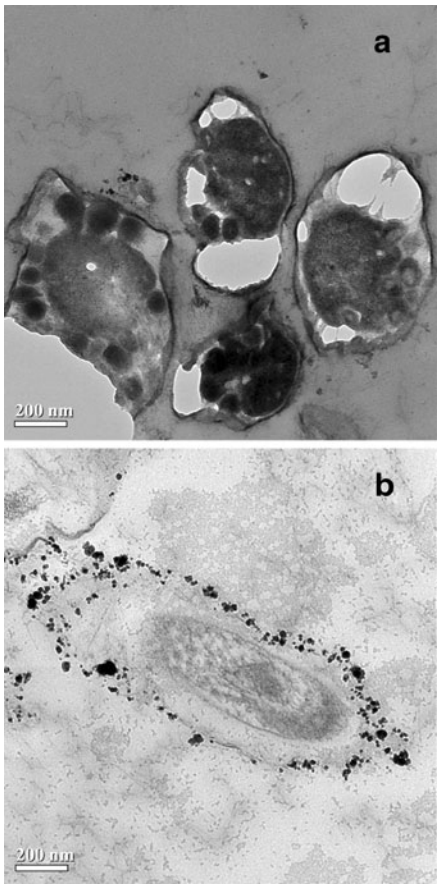

Fig. 2 TEM images of cells from the experiment at an AgNP concentration of 10 mg/L

inhibition in this study. A further work on the point should be considered.

3.4 TEM Observation

Figure 2 presents the TEM image of the NAS samples from an experiment at an AgNP concentration of 10 mg/L. The cells from the experiment at the highest AgNPs applied were chosen as an example. In Fig. 2a, it is obvious that AgNPs damaged the microbial cells. The cells were broken inside (Fig. 2a). This is similar to previous studies that reported about cell breakage after exposing in toxic substances (Andra et al. 2007; Wu et al. 2010). The toxic substance attached on cell membrane and wall attributing to their abrasion (soft edge). After that, outer layer was detached, causing the cell death eventually. The result was well supported by the ammonia oxidation activities discussed earlier. Silver nanoparticles caused cell damage and affected cell functionality.

Figure 2b presents the clump of AgNPs attached on a layer around the cells. This is well described along with the kinetic result mentioned earlier. In previous studies, AgNPs at very low concentrations (up to 1 mg/L) could significantly lower nitrification activities up to 90 % (Choi et al. 2008). However, in this study, AgNPs at the concentrations up to 10 mg/L still lowered the ammonia oxidation activities only 50 %. Even though the result from this study is only on ammonia oxidation activities, the inhibition should be similar as stated previously. Based in Fig. 2b, a protection layer around the cells could be extracellular polymeric substance (EPS). Normally, EPS was produced from cell lysis and aggregated to the organic matter including the cells (Sheng et al. 2010). In this study, AgNPs may cause cell lysis. The EPS then produced and formed layer all over the cells (including live and dead cells). In the live cells, this layer could lessen the cell-AgNP contact resulted in the reduction of nitrification and ammonia oxidation inhibition. However, the result discussed was just preliminary. The continued work on the component analysis of the layer should be performed for better clarification.

4 Conclusions

Influence of AgNPs on ammonia oxidation was investigated by using respirometric method. The kinetic parameter, K_s, without AgNPs was 15.88 mg N/L, while K_i was 5.5 mg/L. Silver nanoparticles partially influenced ammonia oxidation in the manner that higher AgNPs resulted in higher inhibition of respiration rates. There was the interacting effect of AgNP and ammonia concentrations on ammonia-oxidizing activities. Increasing AgNP concentrations from 0.25 to 10.00 mg/L resulted in higher inhibition level of ammonia oxidation about 4–50 %. The inhibition was evident from the cell damage by AgNPs. This result could be used for further practice, either for design or operation of wastewater treatment system. Future studies on the role of microbial community and EPS in ammonia oxidation under the presence of AgNPs were recommended for insight information.

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