Pathway and mechanism of nitrogen transformation during composting: Functional enzymes and genes under different concentrations of PVP-AgNPs

Guangming Zeng [a,b,*], Lihua Zhang [a,b], Haoran Dong [a,b], Yaoning Chen [a,b], Jiachao Zhang [c], Yuan Zhu [a,b], Yujie Yuan [a,b], Yankai Xie [a,b], Wei Fang [a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha, 410082, PR China

[b] Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Ministry of Education, Changsha, 410082, PR China

[c] College of Resources and Environment, Hunan Agricultural University, Changsha, 410128, PR China

[*] Corresponding author. Address: College of Environmental Science and Engineering, Hunan University, Changsha, 410082, PR China. Tel.: +86 731 88822754; fax: +86 731 88823701.

E-mail address: zgming@hnu.edu.cn (G. Zeng).

Abstract

Polyvinylpyrrolidone coated silver nanoparticles (PVP-AgNPs) were applied at different concentrations to reduce total nitrogen (TN) losses and the mechanisms of nitrogen bio-transformation were investigated in terms of the nitrogen functional enzymes and genes. Results showed that mineral N in pile 3 which was treated with AgNPs at a concentration of 10 mg/kg compost was the highest (6.58 g/kg dry weight (DW) compost) and the TN loss (47.07%) was the lowest at the end of composting. Correlation analysis indicated that TN loss was significantly correlated with *amoA* abundance. High throughput sequencing showed that the dominant family of ammonia-oxidizing bacteria (AOB) was *Nitrosomonadaceae*, and the number of Operational Taxonomic Units (OTUs) reduced after the beginning of composting when compared with day 1. In summary, treatment with AgNPs at a concentration of 10 mg/kg compost was considerable to reduce TN losses and reserve more mineral N during composting.

Key words: PVP-AgNPs; Composting; Nitrogen transformation; Functional enzymes; Functional genes

1. Introduction

Sewage sludge (SS) is an essential by-product during wastewater treatment process. With the rapid expanding of wastewater treatment plants (WWTP), the production of SS also increased dramatically. Researches reported that the averagely annual production of SS in WWTP of China was 30 million tons and increased by more than 13% between 2007 and 2015 (Cai et al., 2016). SS is favorable for recycling to agriculture because of considerable fractions of nitrogen, phosphorus and other fertilizing elements in it. However, SS can not be directly reused as fertilizer for the high quantities of pathogenic microbes, organic micro-pollutants and toxic heavy metals, which are strictly controlled by the legislation of SS applications (Zeng et al., 2013a). Consequently, the development of environmentally friendly and sustainable measures for disposal of SS has received much fervent attention (Vogel et al., 2011; Zeng et al., 2013b).

Composting is a cost-efficient and social preferred approach for solid waste management. During SS composting, unstable and complex organic matters are degraded into stable and humus-like substances without toxic effects and the composted SS is preferred as soil conditioner and organic fertilizer for agriculture (Huang et al., 2008; Tang et al., 2008). As a critical factor for evaluating the quality of final composts, nitrogen bio-transformation during SS composting is complex, including volatilization, mineralization, immobilization, nitrification and denitrification, and the loss of nitrogen is mainly caused by the release of NH_3, N_2O, N_2 and NO_x (Zhang et al., 2017). Many researchers have been exploring more

53 effective methods to reduce nitrogen loss during SS composting. Since the high
54 concentration of nitrogen and low C/N ratio would lead to excessive ammonia
55 volatilization (Malińska et al., 2014; Yang et al., 2010), extra carbon sources (e.g.
56 sawdust, straw, paper, biochar) were introduced to improve the physico-chemical
57 properties of composting materials (Malińska et al., 2014). Other additives like lime
58 and zeolite were also applied to alleviate the acidic pH and reduce the release of NH_3
59 and N_2O (Cheng et al., 2016).
60 The development of nanotechnology has brought huge increases in production and use
61 of engineered nanoparticles (ENPs) (Xu et al., 2012). The project of Emerging
62 Nanotechnologies at the Woodrow Wilson International Center for Scholars reported
63 that consumer products containing ENPs increased by 521% between 2006 and 2011
64 (Feng et al., 2010; Gong et al., 2009; Impellitteri et al., 2013). Due to the
65 antimicrobial properties, silver nanoparticles (AgNPs) are applied to various
66 consumer products, such as food containers, sporting goods, clothing, softeners and
67 detergents (Kim et al., 2010). Many researches have tried to illustrate the relationships
68 between AgNPs and microorganisms. In soil ecosystem, several studies suggested that
69 denitrifying bacteria and denitrification were easily vulnerable to AgNPs (Mishra et
70 al., 2015). It was found that NO_3^--N reduction was retarded even by low concentration
71 of AgNPs in sequencing batch reactors, and NO_2^--N kept the same low levels and this
72 might be due to that AgNPs inhibited the activity of nitrate reductase, while brought
73 less adverse effects on nitrite reductase under low level (Hu et al., 2011; Zhang et al.,
74 2016c). Previous literatures reported that nitrifying bacterial activity was decreased by

86% under 1 mg/L AgNPs (Choi et al., 2008; Zhang et al., 2007), while other studies found that nitrification was decreased by 41.4% at the same concentration of AgNPs in an activated sludge treatment system (Fan et al., 2008; Liang et al., 2010). These previous studies provided deep insights into the impacts of AgNPs on nitrogen cycling and the related microorganisms. However, few researches linked the nitrogen transformation with the functional enzymes and genes simultaneously during composting with the existence of PVP-AgNPs and few researches studied the diversity of the functional genes which shown significant correlation with total nitrogen (TN) losses.

The present study was conducted to investigate the impacts of PVP-AgNPs under different concentrations on nitrogen bio-transformation during composting. The changes of functional enzymes activities and genes abundances for nitrogen bio-transformation were determined, since these data help explain the nitrogen changes. Also, the bacterial *amoA* gene which was significantly correlated with TN losses was sequenced to study the diversity of ammonia-oxidizing bacteria (AOB). This research represents the few researches which reveal the impacts of AgNPs on nitrogen transformation by integrating the analyses of enzyme activities and molecular level of nitrogen cycling microbial communities.

2. Experimental section

2.1. PVP-AgNPs synthesis and characterizations

Polyvinylpyrrolidone coated AgNPs (PVP-AgNPs) was chosen since this kind of AgNPs can maintain colloidal stability in composting systems where the environment

97 is complex and the ionic strength is high with high valence background electrolytes
98 (Gitipour et al., 2013). Briefly, 5×10^{-3} M $AgNO_3$ was dropwise (~1 drop per second)
99 added into a vigorously stirring solution of 1% PVP and 2.5×10^{-3} M $NaBH_4$ at a
100 volume ratio of 1:3 and this procedure was conducted in a condition of ice bath. In
101 addition, the synthesized PVP-AgNPs were purified three times using 1 kDa dialysis
102 membranes to clear the rest of reactants. This purification method could prevent the
103 solutions drying, aggregating or oxidizing, and the concentration of AgNPs was kept
104 the same by replacing the excess by-products with water.
105 UV-vis absorption spectrum analysis was performed using a Shimadzu UV-2550
106 (Japan) at a wavelength range of 300-800 nm to confirm the successful synthesis of
107 AgNPs. Transmission electron microscopy (TEM) and energy dispersive X-ray
108 spectrophotometer (EDX) analysis were carried out to investigate the morphology and
109 further confirm the formation of AgNPs. Samples for TEM and EDX were prepared
110 by adding a drop of AgNPs solution on a carbon coated copper grid and then air-dried
111 at room temperature. The TEM and EDX photographs were captured by JEOL,
112 JEM-2100F complemented with EDX and the accelerating voltage was 200 kV.
113 Hydrodynamic diameter (HDD) were determined using Zetasizer Nanoseries
114 (Malvern Instruments, UK) according to the theory of dynamic light scattering (DLS)
115 with a 633 nm laser source and a 173^{o} detection angle and the detection range was
116 from 1 nm to 10 μm.
117 2.2. Materials preparation, composting and sampling
118 Sewage sludge was obtained from Yuelu wastewater plant of Changsha, China and

119 then it was air-dried and sieved through the 100-mesh screen. Rice straw, collected
120 from suburb of Changsha, China, was also air-dried and cut into 10~20 mm lengths. It
121 is a kind of typical agricultural waste difficult to degrade. As the easily degradable
122 materials to assist the composting processes, vegetables were chopped into 10~20 mm
123 lengths after being air-dried. To improve the initial carbon to nitrogen (C/N) ratio,
124 bran was air-dried and added into the composting piles. The characteristics of the raw
125 materials were presented in Table 1. Initial moisture content was regulated to 65% and
126 C/N ratio was adjusted to about 25 (Gitipour et al., 2013; Huang et al., 2017a) by
127 blending sewage sludge, rice straw, vegetables and bran at a weight ratio of 22:36:5:5.
128 Five indoor composting piles were set up including pile 1 (without AgNPs) and pile
129 2~5 which were treated with AgNPs at concentrations of 2, 10, 20, 30 mg/kg compost,
130 respectively. The composting processes lasted for 60 days during which most of
131 complex organic matters were degraded to stable substances. The piles were turned
132 daily during the first 14 days and weekly afterwards. Solid samples were collected
133 from three different locations of the piles and homogenized before stored at 4 ^{o}C for
134 determinations of physico-chemical parameters and -20 ^{o}C for DNA extraction.
135 2.3. Physico-chemical parameters and nitrogen functional enzymes determination
136 Temperature was recorded from five different positions of the composting piles using
137 a thermometer. The NH_4^+-N, NO_3^--N, NO_2^--N concentrations were determined
138 according to the methods in previous studies (Zhang et al., 2017) by shaking the
139 samples in 2 M of KCl solution at a ratio of 1:50 (w/v) for 1 h to extract the mineral N.
140 Mineral N equaled to the sum of NH_4^+-N and NO_3^--N. Samples for TN determination

were ground after being dried at 105 ^{o}C for 24 h and analyzed using Kjeldahl digestion analysis. The TN losses were calculated according to the following formula (Zhang et al., 2017):

$$\text{TN loss (\%)} = 100\text{-}100[(X_1 N_n) / (X_n N_1)] \quad (1)$$

in which N_1 and N_n represented the total nitrogen concentration on initial and each corresponding day, and X_1 and X_n represented ash content on initial and each corresponding day, respectively. The activities of nitrate reductase (NR), nitrite reductase (NIR), ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR) were measured referring to previous literatures (Zheng et al., 2011) (see Supplementary materials).

2.4. DNA extraction

The total genomic DNA was extracted from 0.3 g samples of each composting pile using the E.Z.N.A. ® Soil DNA Kit (OMEGA Bio-Tek, Inc., Norcross, GA, USA). The extraction was conducted according to the manufacturer's instructions. The quality and concentration of DNA extraction were determined using a NanoDrop (Thermo Scientific, Wilmington, DE, USA). The purification of crude DNA was performed using the Universal DNA Purification Kit (TIANGEN, China) according to the manufacturer's instructions for use. Three parallel extractions were performed for each compost sample.

2.5. Quantification of nitrogen functional genes

The abundances of functional genes, encoding the key enzymes involved in nitrification (*amoA* and *nxrA* encoding ammonia monooxygenase and nitrite

oxidoreductase, respectively) and denitrification (*narG*, *nirK*, *nirS*, and *nosZ* encoding the membrane-bound nitrate reductase, copper-containing nitrite reductase, cd_1-containing nitrite reductase, and nitrous oxide reductase, respectively), were used to represent the abundances of functional microbes (Zhi et al., 2014). The quantitative PCR (qPCR) was conducted on an iCycler IQ5 Thermocycler (Bio-Rad, USA) in a system of 10 μL of 2 × SuperRedal PreMix Plus (with SYBR Green I), 0.4 μL of each primer (50 μM), 1 μL of template DNA, and 8.2 μL of sterile ultrapure water (see Supplementary materials). All the qPCR reactions set up the negative control by replacing the unknown DNA sample with sterile ultrapure water, and three parallel qPCR analyses were performed for each sample. The gene copies obtained from qPCR reaction were converted to copies per gram of dry weight (DW) compost.

2.6. High throughput sequencing and sequence analysis

Compost samples were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) for sequencing of bacterial functional gene *amoA* (see Supplementary materials). The raw sequences were submitted to NCBI with BioProject ID PRJNA385265.

2.7. Statistical analyses

All analyses of parameters apart from temperature which was determined from five different positions of the composting piles were performed in triplicate. For the evaluation of differences between composts from different piles, least significance difference (LSD) test was conducted at 95% confidence level using SPSS 19.0. If $P >$ 0.05, the difference between piles was thought to be statistically insignificant. Pearson

correlations coefficients were calculated using MATLAB R2013a to assess the relationships between TN losses and the functional genes of N transformation, and a correlation matrix (7 × 7) was created.

3. Result and discussion

3.1. PVP-AgNPs characterizations

The absorption peak around 400 nm suggested the formation of AgNPs, and no peaks else were found less than 390 nm indicating that there were not impurities (e.g. Ag^{+}) in the AgNPs solution (see Supplementary materials). Spherical particles were found distributed evenly on the carbon coated copper grid and the average size was about 6 nm. And the EDX analysis diagram further suggested that the particles in TEM graph were the PVP-coated AgNPs. The average HDD of the synthesized PVP-AgNPs was about 8.5 nm. The little bit of difference between TEM size and HDD size once be reported that it might be caused by the following: (1) the process of drying TEM samples might lead to the shrinkage of the PVP molecule; (2) the aggregated PVP-AgNPs were not dispersed fully during sonication and resulted in larger HDD size; and (3) the PVP coated more than one AgNPs at the same time (El Badawy et al., 2010).

3.2. Temperatures of composting piles

As well known, temperature was an essential parameter to evaluate the performance of decomposition of organic matter during composting process (Huang et al., 2017a; Wang et al., 2013). Since the highest temperature in pile 5 was lower than 50 ^{o}C, all other parameters of pile 5 were not presented in this literature. The time courses of

207 temperatures in other four composting piles were similar to many patterns which
208 included three typical phases (mesophilic, thermophilic and cooling stage) (see
209 Supplementary materials). The significant differences (P<0.05) between all treatments
210 were observed during the first 12 days and this was mainly caused by the population
211 succession of mesophilic-thermophilic microorganisms. The temperatures rose
212 dramatically during the early phases of composting due to the decomposition of easily
213 degradable organic matter by microbes, and reached to the highest on day 5 for pile 1
214 (59.6 ^{o}C), day 7 for pile 2 (55.8 ^{o}C) and pile 3 (60.4 ^{o}C), and day 4 for pile 4 (61 ^{o}C),
215 respectively. It was fastest to achieve highest temperature in pile 4 and the
216 thermophilic phase of pile 4 lasted for 11 days, while 10 days in other piles. The
217 temperatures over 50 ^{o}C were continued for more than 5 days, which was the
218 requirement of Chinese National Standard and the 3-days duration of temperatures
219 over 55 ^{o}C in all composting piles met the minimum requirement for destroying all
220 pathogens (Zhang et al., 2011). In terms of temperature, the main impacts of AgNPs
221 were the time when the highest temperatures reached and the values of the highest
222 temperatures, and all treatments presented different levels of temperatures during
223 thermophilic phase. This suggested that AgNPs might have some impacts on
224 heterotrophic microorganisms, even promote the activities of heterotrophic
225 microorganisms.
226 3.3. Nitrogen transformations
227 Nitrification and denitrification are two critical pathways of nitrogen transformation.
228 The fluctuation of NH_4^+-N in composts was an important implication for the nitrogen

transformations and NH_3 release (Jiang et al., 2015). In this study, NH_4^+-N dominated the inorganic nitrogen during composting, and the concentrations of NH_4^+-N in all treatments increased dramatically during early stages of composting because of ammonification, and reached peak value on day 5 in pile 1 and pile 3, and on day 10 in pile 2 and pile 4, respectively (Fig. 1a), this trend was similar to other literatures (Wang et al., 2013).The maximum NH_4^+-N concentration was detected in pile 4 (9990.04 mg/kg dry weight (DW) compost) into which the highest concentration of AgNPs (20 mg/kg compost) was added. The delayed peak time and greater peak value of NH_4^+-N in pile 4 might be due to that the addition of AgNPs at a concentration of 20 mg/kg compost slowed down the mineralization of organic nitrogen and inhibited transformation of NH_4^+-N to NO_2^--N during early stage of composting. Then, NH_4^+-N concentrations in all treatments decreased rapidly till day 15, which might be due to the facts that high temperature and pH provided favorable conditions for NH_3 emission, and immobilization by nitrogen fixing microbes (Wang et al., 2016b). Thereafter, it continually decreased slowly till the end of composting. The NH_4^+-N concentrations of pile 1, pile 2, pile 3 and pile 4 were 5482.44, 4655.25, 6334.96 and 5822.52 mg/kg DW compost at the end of composting, respectively. It was 1.09~1.36 times higher in pile 3 than other piles. One-way analysis of variance (ANOVA) presented a significant difference among the four treatments (F=64.9, P<0.01), and LSD tests indicated highly significant differences between pile 3 and other piles (P<0.05) at the end of composting.

Similar to NH_4^+-N, NO_3^--N contents increased rapidly during the first several days of

composting (Fig. 1b) owing to alkaline pH (see Supplementary materials) which provided favorable conditions for transformation of NO_2^--N to NO_3^--N by nitrite oxidizing bacteria (NOB) (Zhang et al., 2016b). The NO_3^--N in pile 3 peaked fastest to 4791.34 mg/kg DW compost, while the peak value of NO_3^--N in pile 4 (5976.68 mg/kg DW compost) was the highest (P<0.05). Then NO_3^--N concentrations presented downtrends, and pile 3 showed significant lower NO_3^--N content at the end of composting. According the previous study (Bustamantea et al., 2008), the exposure of unavailable NH_4^+-N to microbes could suppress the nitrification and lead to lower NO_3^--N.

As shown in Fig. 1c, the content of NO_2^--N was relatively low as it was the intermediate product generated during nitrification by ammonia-oxidizing microbes. During the first three days, NO_2^--N were not detected in all piles and increased rapidly since day 4 to peak values on day 10, then presented short periods of decline. Thereafter, the trends of NO_2^--N were divided into two groups: pile 1 and pile 2 continually decreased till the end, while pile 3 and pile 4 increased slowly. This suggested that higher concentrations of AgNPs would inhibit conversions of NO_2^--N to NO_3^--N.

The time courses of total mineral nitrogen (Fig. 2a) were similar to NH_4^+-N, mainly because that NH_4^+-N dominated the mineral nitrogen. At the beginning, mineral nitrogen contents increased dramatically though the TN declined (Fig. 2b). This might be on account of the transformation of organic nitrogen to mineral nitrogen and losses of nitrogen as NH_3, N_2O, NO_X, etc. during this process. Afterwards, concentrations of

mineral nitrogen in all treatments decreased till the end of composting. With organic nitrogen was mineralized, the activities of microorganisms were thought to be increased, which induced fierce competitions for nitrogen substrates and the consequent immobilization of mineral nitrogen (Azeez et al., 2010). The mineral nitrogen concentrations were 6.2, 5.22, 6.58, and 6.18 g/kg DW compost in pile 1 ~ pile 4 in the end, respectively. The highest mineral nitrogen content was detected in pile 3 and the loss of TN in pile 3 was the lowest (P<0.05) (Fig. 2c) at the end of composting. Comparing with the initial TN, the lowest TN loss in pile 3 was 13.76% which was lower than the control pile (17.74%) by 22.4%. While in the previous study, the lowest nitrogen loss (19.72%) was detected in the pile which was treated with 10% biochar and 10% zeolite during pig manure composting (Wang et al., 2017). Therefore, the impact on the conservations of nitrogen and improvements of N availability was the most remarkable when pile was treated with AgNPs of 10 mg/kg compost in this study.

3.4. Activities of key enzymes

Biological transformation of nitrogen is critically dependent on the activities of key enzymes. Many previous literatures reported that nitrification and denitrification are two key pathways in nitrogen bio-transformation (Zheng et al., 2011). AMO and NOR are two key enzymes for nitrification, whereas NR and NIR are crucial to denitrification (Zheng et al., 2011). As shown in Fig. 3a, the activity of AMO in pile 3 was the lowest since day 15, indicating that the existence of AgNPs at a concentration of 10 mg/kg compost inhibited the activity of AMO. It was significantly lower than

295 pile 2 during the whole composting but was insignificantly lower than pile 1 and pile
296 4 after day 26. The presence of AgNPs at a concentration of 2 mg/kg compost
297 stimulated the activity of AMO. These observations further explained the higher
298 NH_4^+-N in pile 3 at the end of composting (Fig. 1a). Fig. 4b showed that the addition
299 of AgNPs at a concentration of 20 mg/kg compost inhibited the activity of NOR
300 which was significantly higher in pile 1 than pile 4, while insignificantly higher than
301 pile 2 and pile 3. These observations were consistent with the higher concentrations of
302 NO_3^--N in pile 1 since the day 26 of composting processes (Fig. 1b). However the
303 highest activity of AMO in pile 2 did not result in higher concentration of NO_2^--N (Fig.
304 1c), and this might be due to the relatively higher activities of NOR and NIR (Fig. 3)
305 than pile 3 and pile 4, and lower activity of NR. As shown in Fig. 3c and 3d, the
306 activity of NR was higher in pile 1, and that of NIR were higher in pile 1 and pile 2,
307 resulting in lower concentration of NO_2^--N in pile 1 and pile 2 during the cooling and
308 maturation stages. It was in accordance with other literatures that found higher levels
309 of AgNPs inhibited the activities of NR and NIR (Zhang et al., 2016c).
310 3.5. Quantification of nitrogen functional genes
311 The genes (*amoA*, *nxrA*, *narG*, *nirK*, *nirS* and *nosZ*) were quantified for all piles
312 during composting (Fig. 4). The highest abundances of *amoA* which is the marker of
313 aerobic oxidation of NH_4^+-N to NO_2^--N were determined at the beginning of
314 composting in all piles and then decreased rapidly. The copy numbers of *amoA* in pile
315 3 were restrained by AgNPs since day 15 till the end of composting, resulting in
316 higher concentration of NH_4^+-N in pile 3 during the same period. Among the four

piles, the average copy number of *amoA* in pile 3 was the lowest and the order of other three piles was: pile 4 > pile 1 > pile 2. This indicated the vulnerability of AOB to AgNPs at a concentration of 10 mg/kg compost, while it was interesting to found that the *amoA* copies increased under a concentration of 20 mg/kg compost. Other interesting phenomena were found by previous studies that the impact on *amoA* was more pronounced in 35-nm AgNPs treated samples than 5-nm AgNPs treated samples (Yang et al., 2014). As shown in Fig. 4b, higher *nxrA* copies were detected in pile 1 during the whole composting period, which suggested that NOB was vulnerable to AgNPs and it was more sensitive in pile 3 treated with 10 mg/kg compost AgNPs. The order of average *nxrA* abundances in four piles was pile 1 > pile 2 > pile 4 > pile 3. The similar temporal variation trends of *nxrA* and *amoA* in pile 1 might be due to the similar survival conditions that both AOB and NOB needed aerobic conditions and were autotrophic, and the ecological relation that AOB supplied NO_2^--N by oxidizing NH_4^+-N to NO_2^--N for NOB to transform NO_2^--N to NO_3^--N (Zhi et al., 2014). However, the changes of *nxrA* were not consistent with that of *amoA* in other three piles since the disturbance of AgNPs.

The detectability of *narG*, *nirK*, *nirS*, and *nosZ* suggested the occurrence of denitrification during composting of all treatments. *NarG* is the well-known representative encoding membrane-bound nitrate reductase to perform the reduction of NO_3^--N to NO_2^--N (Lopez-Gutierrez et al., 2004). The highest abundance of *narG* of each pile was detected on day 26 or 38 and the time courses of *narG* gene in all composting piles exhibited an increase from the beginning to day 26 or 38 and then

339 decrease till the end of composting processes (Fig. 4c). Among the four piles, *narG* in
340 pile 4 was the highest especially from day 26 to the end of composting ($p < 0.05$). The
341 order of average *narG* abundance was pile 4 > pile 3 > pile 2 > pile 1, suggesting that
342 AgNPs had a positive impact on *narG* gene and the impact was more pronounced
343 under exposure of higher AgNPs concentration. This was similar to other researches
344 that found AgNPs did not significantly influence the expression of *narG* and even
345 could stimulate the survival of microbes (Fajardo et al., 2014; Xiu et al., 2012). *NirK*
346 and *nirS* are two functional genes involved in the second denitrification step, namely
347 the reduction of NO_2^--N to NO and this is the first stage for production of gas in
348 denitrification (Huang et al., 2017b). The abundance of *nirK* in all piles increased till
349 day 26 or 38 and then decreased till the end of composting (Fig. 4d). Order of average
350 *nirK* copies was pile 2 > pile 1 > pile4 > pile 3 and that of *nirS* was pile 2 > pile 4 >
351 pile 3 > pile 1. While for *nirS* gene abundance, it decreased rapidly in all piles during
352 the first 5 days and increased continuously till day 38, and decreased again till the end
353 (Fig. 4e). The average copy number of *nirK* was about 2~4-fold higher than that of
354 *nirS*, similar to other studies (Wang et al., 2013). The higher *nirK* gene abundance
355 indicated that it was more tolerant of AgNPs, and it also suggested that *nirK* was
356 dominant in nitrite reduction step and the main contributor to generation of
357 greenhouse gas (NO) (Wang et al., 2016a). Since both *nirK* and *nirS* participated in
358 the second step of denitrification, the total abundances of the two genes were
359 calculated to evaluate the potential for transformation of NO_2^--N to NO (Yan et al.,
360 2003). The variation tendencies were similar to that of *nirK* with increasing from

beginning to day 26 or 38, and then decreasing till the end of composting. The average total abundances of *nirK* and *nirS* in all piles arranged in the order of pile 2 > pile 1 > pile 4 > pile 3, which was in accordance with the single *nirK* gene and was similar with that of *narG*. This might be due to the higher *nirK* gene abundance than *nirS*, and on the other hand, *narG* codase catalyzed the transformation of NO_3^--N to NO_2^--N, providing substrate NO_2^--N for *nirK* codase and *nirS* codase to convert it into NO so that their abundances might be influenced by *narG*. *NosZ* is often regarded as the marker for the last step of denitrification in which N_2O was conversed into N_2. As shown in Fig. 4f, the abundance of *nosZ* in each pile exhibited different change tendencies. In pile 1, the highest copy number was detected on day 38 with constant fluctuations during composting process. In pile 2 and pile 3, *nosZ* abundance increased till day 38 and then decreased till the end of composting. While in pile 4, it decreased from day 1 to day 5 followed by increasing till day 15, and then decreased till day 38 which was followed by an increase till the end when the copy number was the highest. According to the comparison among the four piles, the average abundance of *nosZ* in pile 4 was highest. The order of remaining three piles were pile 3 > pile 2 > pile 1. This indicated that the stimulation to *nosZ* was more obvious with higher AgNPs.

3.6. Correlation between TN losses and nitrogen functional genes

As shown in Fig. 2c, TN losses in pile 3 were the lowest compared with other three piles. So, correlation coefficients were determined between TN losses and nitrogen functional genes in pile 3 (Fig. 5). The results of correlation analysis showed that TN

loss in pile 3 was most significantly and negatively correlated with the abundance of *amoA* (r = -0.9317, $P < 0.01$). This can be interpreted as that the *amoA* gene encodes AMO to convert NH_4^+-N into NO_2^--N, which results in the decrease of NH_4^+-N. And the lower concentration of NH_4^+-N will reduce NH_3 emissions and TN losses (Zhang et al., 2011). Song et al. (2016) reported a negative correlation between NH_3 and ammonia-oxidizing archaea (AOA), but a positive relationship between NH_3 and AOB. Additionally, both AOA and AOB may be inhibited under high concentrations of NH_3 (Jung et al., 2014).

3.7. Diversity of ammonia-oxidizing bacteria (AOB)

According to the result of the correlation analysis between TN losses and functional genes in pile 3, the diversity of *amoA* gene in pile 3 was studied by sequencing. Totally, 92917 sequences of bacterial *amoA* with sequences of 11919, 19406, 12425, 18835, 15705, and 14627 for each sample respectively, were obtained after removal of low quality sequences (< 20), sequences smaller than 50 bp, primer chimeras, non-ribosomal sequences and sequence tags (Table 2). The average length of sequences was about 469 bp. All sequences were clustered into 45 OTUs in total. Highest number of OTUs was detected in samples from day 1 (27 OTUs), while the lowest number was 6 which was observed in samples from day 5 (6 OTUs), and then it increased continuously to 12 OTUs at the end of composting. The calculation of Chao1 and ACE which were the indices of community richness revealed that AOB species were the richest at the beginning of composting. Also, the Shannon index of AOB in each sample suggested that the diversity of AOB from day 1 was the highest,

405 while it was lowest in samples from day 5. Both the richness and diversity of AOB
406 community began to increase from day 5, which was similar to other studies that only
407 found some weak denaturing gradient gel electrophoresis (DGGE) bands during
408 thermophilic phase of agricultural composting (Zhang et al., 2016a).
409 Fig. 6 revealed the relative abundances of phylotypes at OTUs and family level in
410 each sample from pile 3 during composting. Fig. 6a showed that OTU 25 (44.61% ~
411 72.561%), OTU 34 (7.84% ~ 20.91%) and OTU 8 (0.04% ~ 8.96%) existed during the
412 entire composting process, and OTU 25 dominated AOB community with slight
413 reduces during composting process. Also, OTU 34 played an important role during the
414 ammoxidation process. However, some kinds of OTUs (OTUs 14 and OTUs 4) which
415 existed at the beginning of composting disappeared under the exposure to AgNPs. As
416 shown in Fig. 6b, the predominant family *Nitrosomonadaceae* which was affiliated to
417 *Proteobacteria* and *β-Proteobacteria* at phylum and class level respectively ranged
418 from 71.63% to 98.44% in all samples in terms of relative abundance. Previous
419 studies also reported that *Proteobacteria* was the most predominant phylum counting
420 for about 7.4%~46.4% of total effective sequences in twelve wastewater treatments
421 systems (Shu et al., 2016). It was once detected that *Proteobacteria* was the largest
422 group which could resist silver nanoparticles (Yang et al., 2014), this was in
423 accordance with the present literature. Lai et al. (2014) found that *β-Proteobacteria*
424 was the absolutely predominant class with relative abundance of more than 90%,
425 while the abundance of *α-Proteobacteria* was less than 7% when about 10 mg/L
426 NO_3^--N was added into membrane biofilm reactor in their study. The family of

427 *Nitrosomonadaceae* could be further divided into *Nitrosomonas*, *Nitrosospira* and
428 some unclassified genuses. It was once reported that *Nitrosomonas* was the
429 predominant AOB, while the abundance of *Nitrosospira* was relatively low in the
430 landfill leachate treatment system under high organic load (Remmas et al., 2016). On
431 the other hand, the genus *Nitrosomonas* detected in the present study was related to
432 *Nitrosomonas sp.* Nm 84, *Nitrosomonas sp.* Is 79A3, *Nitrosomonas eutropha* and
433 some unclassified species with 97% similarity at least. As for *Nitrosospira*, it was
434 closely associated with *Nitrosospira briensis*, which has also been suggested that
435 *Nitrosospira briensis* was the representative belonging to *Nitrosospira* genus that was
436 previously found in land leachate treatment plants (Urakawa et al., 2015; Yapsakli et
437 al., 2011). However, the species of *Nitrosomonas sp.* Is 79A3, *Nitrosomonas eutropha*
438 and *Nitrosospira briensis* were undetectable since day 5 of the composting process,
439 which indicated that these three AOB species were easily vulnerable to AgNPs at
440 concentration of 10 mg/kg compost. The results of unweighted PCoA analysis on
441 species level reveled that most of samples possessed different species (see
442 Supplementary materials). Samples of day 5, 15, 26, 38 were grouped together with
443 high similarity between day 5 and 38, while samples of day 1 and day 60 showed
444 quite different species compared with other samples.

445 **4. Conclusion**

446 In summary, the results of the present study showed that concentration of mineral N
447 was the highest and the TN loss was the lowest at the end of composting when
448 treating compost with AgNPs at a concentration of 10 mg/kg compost. The results

449 also revealed that AgNPs brought different impacts on functional enzymes and genes
450 for nitrogen bio-transformation. These results provided a deeper insight into the
451 bio-transformation of nitrogen and a new method to reduce TN losses.

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Figure captions:

Figure 1. Changes of (a) NH_4^+-N concentration; (b) NO_3^--N concentration and (c) NO_2^--N concentration during composting processes. Mean values and standard deviations (n = 3) were shown.

Figure 2. Changes of (a) mineral N, (b) TN concentration, and (c) TN losses during composting processes. Mean values and standard deviations (n = 3) are presented.

Figure 3. Activities of functional enzymes during composting processes. The unit of activities is μmol nitrite / (min · mg protein). (a) AMO; (b) NOR; (c) NR; (d) NIR. Mean values and standard deviations (n = 3) were shown.

Figure 4. Changes of functional genes copy numbers during composting processes. Mean values and standard deviations (n = 3) were shown.

Figure 5. Relationships between TN losses and functional genes (*amoA*, *nxrA*, *narG*, *nirK*, *nirS*, and *nosZ*) of pile 3. Warm color represents positive correlation coefficients, whereas cool color represents negative correlation coefficients, and smaller ovals represent lower correlations, while bigger ovals represent stronger correlations.

Figure 6. Relative abundance of bacterial *amoA* gene at (a) OTUs level; (b) family level.

Table legends:

Table 1. The physico-chemical characteristics of the raw materials (dry weight).

Table 2. Richness and diversity of bacterial *amoA* gene sequences in pile 3 during composting processes.