Who contributes more to N₂O emission during sludge bio-drying with two different aeration strategies, nitrifiers or denitrifiers?

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Abstract Global warming effects have drawn more and more attention to studying all sources and sinks of nitrous oxide (N₂O). Sludge bio-drying, as an effective sludge treatment technology, is being adopted worldwide. In this study, two aeration strategies (piles I and II) were compared to investigate the primary contributors to N₂O emission during sludge bio-drying through studying the evolution of functional genes involved in nitrification (*amoA*, *hao*, and *nxrA*) and denitrification (*narG*, *nirS*, *nirK*, *norB*, and *nosZ*) by quantitative PCR (qPCR). Results showed that the profile of N₂O emission can be divided into three stages, traditional denitrification contributed largely to N₂O emission at stage I (days 1–5), but N₂O emission mainly happened at stage II (days 5–14) due to nitrifier denitrification and NH₂OH accumulation by ammonia-oxidizing bacteria (AOB), accounting for 51.4% and 58.2% of total N₂O emission for piles I and II, respectively. At stage III (days 14–21), nitrifier denitrification was inhibited because sludge bio-drying proceeded mainly by the physical aeration, thus N₂O emission decreased and changed little. The improved aeration strategy availed pile I to reduce N₂O emission much especially at stages II and III, respectively. These results

indicated that nitrifier denitrification by AOB and biological NH₂OH oxidation due to AOB made more contribution to N₂O emission, and aeration strategy was crucial to mitigate N₂O emission during sludge bio-drying.

Keywords Quantitative PCR · Sludge bio-drying · N₂O emission · Nitrifier denitrification · Nitrogen transformation functional genes

Introduction

As a potent and quite sensitive greenhouse gas, nitrous oxide (N₂O) has a 298 times higher effect than that of CO₂ over a hundred-year lifespan (IPCC 2013). This provided an impetus to the need for researches on immediate actions to lessen N₂O emission from all sources and sinks. Waste management and treatment activities are important sources of greenhouse gas (GHG) emissions, i.e., its N₂O emission accounted for 1.50% of total China N₂O emissions in 2005 (NDRC 2013) and less than 2% of total USA N₂O emissions in 2013 (USEPA 2015).

As one of waste management and treatment activities, sewage sludge treatment and disposal has become a rising challenge in China due to strict legal regulations, land shortages, rising costs, and public concern (Wei et al. 2003), e.g., total sludge production had an average annual growth of 13% from 2007 to 2013, and 6.25 million tons dry solids were produced in 2013 (Yang et al. 2015). Existing technologies used for sludge treatment mainly contain composting, landfill, incineration, anaerobic digestion, and bio-drying (Wei et al. 2003; Yang et al. 2015; Maulini-Duran et al. 2013; Yu et al. 2015; Zhang et al. 2015b). The profile of N₂O emission during sludge composting, landfill, and incineration has been widely investigated (Maulini-Duran et al. 2013; Zhang et al. 2015a; Shim et al. 2014), while sludge anaerobic digestion was

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mainly used for methane production and little total nitrogen (TN) was removed. However, the profile and causes of N_2O emission during sludge bio-drying are still unclear. Similar to but different from sludge composting, sludge bio-drying, in which sludge is dried by means of the heat generated by the aerobic degradation of its own organic substances in addition to forced aeration, has been demonstrated as a prospective method of volume reduction and pre-stabilization (Zhang et al. 2015b; Huiliñir and Villegas 2015). Although there is much in common between bio-drying and composting, the essential difference is the process goal (Tom et al. 2016). Composting aims at fully decomposing the biomass, removing odors, and killing pathogens, and the duration of composting can extend to several months. Bio-drying, on the other hand, aims at removing as much moisture as possible in the shortest time. The process duration varies with the waste material type and the system setup, but it typically should last between 2 and 3 weeks. Aeration strategy, due to controlling the pile temperature and supplying oxygen, is not only one of the key factors of sludge bio-drying operation (Villegas and Huiliñir 2014) but also plays an important role in N_2O formation and emission during sludge composting (He et al. 2001). The aeration of sludge bio-drying can be divided into different stages, in which oxygen concentration, microbial activities, and environmental conditions were quite different (Zhang et al. 2015b), and the characteristics of N_2O emission at different aeration stage, thus, might be quite different. For example, Czepiel et al. (1996) found that N_2O emission of sludge composting was closely associated with the compost age, pile depth, pile temperature, and moisture content, and aeration was reported to increase N_2O formation. However, it is well known that sludge bio-drying is quite different from sludge composting (Huiliñir and Villegas 2015), and the information concerning effects of aeration strategy on N_2O emission during sludge bio-drying is little available. In addition, to our best knowledge, there is also no study focusing on nitrogen transformation during sludge bio-drying. Thus, there is of great concern to investigate nitrogen transformation and N_2O emission during sludge bio-drying.

Nitrifiers and denitrifiers are the two principal contributors for N_2O emission during solid waste treatment processes (Wunderlin et al. 2012), and high N_2O emission were reported from a variety of solid waste treatment plants (He et al. 2001). There are mainly three kinds of pathways producing N_2O including hydroxylamine (NH_2OH) oxidation, nitrifier denitrification, and heterotrophic denitrification (Hu et al. 2012; Wunderlin et al. 2012), but N_2O reduction to N_2 by nitrous oxide-reducing microorganisms is the only microbial sink for N_2O (Thomson et al. 2012). It is obvious that the dominant microbial N_2O formation process largely depends on environmental conditions. For instance, Maeda et al. (2010) identified the microbial denitrification as the key pathway of N_2O production in cow manure composting systems through nitrogen

isotope analysis. Sánchez-García et al. (2014) suggested that denitrification was the main pathway of N_2O emission in one soil type (HP), while ammonia oxidation and nitrifier denitrification were the major processes generating N_2O in another soil type (HC). Understanding of the regulation of these processes and the conditions under which they are likely to prevail is thus important for developing targeted mitigation strategies of N_2O emission.

Thus, in this study, two aeration strategies were adopted as previously reported (Zhang et al. 2015b), and the abundance of key nitrogen transformation functional genes involved in microbial nitrification (*amoA*, *hao*, and *nxrA*) and denitrification (*narG*, *nirS*, *nirK*, *norB*, and *nosZ*) were determined using quantitative PCR (qPCR). Then the main objectives of this study were to (1) find out who contributes more to N_2O emission (nitrifiers or denitrifiers) during sludge bio-drying, (2) evaluate whether alterations in the abundance among the different nitrogen transformation functional genes might explain the profile of N_2O emission, and (3) provide a practical base for the mitigation of N_2O emission during sludge bio-drying.

Materials and methods

Process description and aeration strategy of sludge bio-drying

The sludge bio-drying plant located in Shenyang, Liaoning province in China is designed to treat 1000 tons of dewatered sewage sludge at 80% of moisture content (MC) day^{-1} and employs a horizontal agitated solid bed process (HASB) with forced aeration and mechanical turning. There are 96 tunnels in total, and each tunnel is 90 m long, 3 m wide, and 2.5 m tall. According to the aeration condition, each tunnel was divided into seven parts: one pre-mixed area and six different aeration areas (areas A to F, Fig. 1). The operational period of sludge bio-drying was set at 21 days, during which each pile was turned once a day along with the tunnel at a rate of marching 4 m day^{-1} . The size of each pile was 4 m long, 3 m wide, and 2.5 m tall.

Two aeration strategies used in this study were previously reported (Zhang et al. 2015b). Briefly, one aeration strategy for pile I was set as the experiment, which was designed according to the functions of aeration in different areas including oxygen supply, heat dissipation, and water evaporation, and another aeration strategy for pile II was set as the control, which was currently used in the plant. Sludge bio-drying was divided into three stages according to the aeration. The aeration in areas A and B was mainly used to supply oxygen for the microbial community to promote increase of pile temperature. In areas C to E, aeration was used to maintain the pile temperature at less than 60 °C in the thermophilic stage. In area F, aeration was mainly used to evaporate water to meet

Fig. 1 The detail information of the sampling position and area distribution during sludge bio-drying, also the relevant key functional genes involved in the three key kinds of N₂O emissions pathway

including biological NH₂OH oxidation, nitrifier denitrification, and heterotrophic denitrification were located and quantified in this study (modified from Hu et al. 2012)

the requirement that the MC should be less than 40% of the sludge bio-drying product. The primary difference of the aeration between piles I and II was that the aeration rate in area F was increased a lot for pile I, while the aeration rate in other areas decreased or improved, e.g., time control model (min h⁻¹) of piles I and II as A, 6 and 4; B, 6 and 8; and C, 12 and 15; respectively, and the set temperature in temperature control model of piles I and II as 45 °C for A and 55 °C for B. The details are described in Table S1.

Sampling and chemical analysis

Gas samples for N₂O analysis were collected using the static chamber method as previously reported (Zhong et al. 2013). Airtight syringes (60 mL) were used to take gas samples from a rectangular chamber (14.28 L. with a size of 35 × 24 × 17 cm) at the center of each pile by continuous collection four times every 3 min, meanwhile the temporal temperature of the gas was monitored. The N₂O concentrations of the gas samples were analyzed by a gas chromatograph (Agilent 4980, USA) equipped with an electron capture detector (ECD). The detailed operational parameters of the

ECD are as follows: the detector temperature was at 350 °C; the 2-mm inside diameter of the stainless steel column was packed with Porapak Q molecular sieve (80 100 mesh⁻¹); and the carrier gas was an argon 10% methane mixture⁻¹ at a flow rate of 30 cm³ min⁻¹. Venting method was employed for ammonia volatilization analysis (Wang et al. 2004b). Two circular sponges (16 cm in diameter and 2 cm in thickness) were sprinkled evenly using 15 mL phosphate glycerol solution (dilute 50 mL analytical phosphate and 40 mL glycerol to 1000 mL with pure water), and then installed into a vented chamber made of gray round PVC tube (15 cm internal diameter and 10 cm high). The sponge inside the chamber (above the ground about 4 cm) was to absorb NH₃, and the top sponge was used to block up NH₃ from the ambient air. After 1 h sampling, NH₃ in the sponge was extracted with 300 mL of 1 M KCl by oscillation for 1 h, and then measured using the colorimetric method. The calculation details of NH₃ and N₂O flux and accumulated emission were provided in supporting information.

The gas and solids sampling in piles I and II throughout the sludge bio-drying process was simultaneously carried out on days 1, 3, 5, 7, 11, 14, 17, and 21, respectively as shown in

Fig. 1, and details for sampling position of each pile were described in Fig. S1. The moisture content (MC; %) was measured by drying the solid sample in a drying oven at 105 °C until a constant weight was achieved. The composite solid sample was divided into two parts. One part was stored at −20 °C until analysis. The other part was freeze-dried and then ground to pass through a 100 mesh. Aqueous sludge bio-drying extracts were obtained by shaking the samples with Milli-Q water at a ratio of 1:10 (w/v) for 1 h, and then used to determine pH. C/N ratios were measured by an elemental analyzer (Vario EL III, Elementar Analysen System GmbH, Germany) with reference material of sulfonamides. For measurement of inorganic nitrogen including $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and $\text{NO}_2^-\text{-N}$, 3.0 g of solid samples was extracted by 30 mL of 2 M KCl solution for 1 h, and the extracts were then filtered through 0.45- μm membrane filters. The filtrates were used to determine inorganic nitrogen, and concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were analyzed by a colorimetric method, while the $\text{NO}_3^-\text{-N}$ concentration was measured by an ultraviolet spectrophotometric method. To determine concentrations of dissolved organic carbon (DOC), 3.0 g solid sample was extracted by 30 mL of Milli-Q water for 24 h and filtered by 0.45- μm membrane. The filtrates were used to determine DOC by a TOC analyzer (Shimadzu TOC-5000, Japan).

DNA extraction

DNA extraction from each solid sample (0.5 g) was completed using a FAST DNA Extraction Kit (MP Biomedicals, USA), and extracted genomic DNA was detected and quantified using 1% agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, USA), and then stored at −20 °C until use.

Quantitative polymerase chain reaction

The nitrogen functional genes involved in every key step of nitrogen transformation (Fig. 1) and bacterial 16S ribosomal RNA (rRNA) were quantified. The gene copies of the key enzymes involved in nitrification (*amoA*, *hao*, and *nxrA* encoding ammonia monooxygenase, hydroxylamine oxidoreductase, and nitrite oxidoreductase, respectively), nitrate reduction (*narG* encoding membrane-bound nitrate reductase), and denitrification (*nirK*, *nirS*, *norB*, and *nosZ* encoding the copper nitrite reductase, cd1 nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively) were determined using qPCR during sludge bio-drying to determine their dynamic population shifts. The plasmids containing these specific genes were manufactured by Zhejiang Tianke Biotechnology Company (Zhejiang, China). The standard samples were diluted to yield a series of 10-fold concentrations and subsequently used for qPCR standard curves. The 25- μL PCR reaction mixtures contained 12.5 μL SYBR Green qPCR Super-Mix-UDG with Rox (Invitrogen, USA),

0.5 μL each of 10 μM forward and reverse primers, 10.5 μL DNA-free water, and 1.0 μL standard plasmid or DNA extract. The thermo-cycling steps for qPCR amplification were as follows: (1) 50 °C, 2 min; (2) 95 °C, 5 min; (3) 95 °C, 20 s; (4) annealing temperature, 30 s; (5) 72 °C, 31 s; (6) plate read, go to (3)–(5), 39 more times; and (7) melting curve analysis, 60 to 95 °C, 0.2 °C read^{-1} . The reaction was conducted using an ABI real-time PCR system 7500 (ABI, USA). The specificity was assured by the melting curves and gel electrophoresis. Each gene was quantified in triplicate with a standard curve and negative control. The primers, annealing temperature used in qPCR, and the corresponding amplification efficiencies are summarized in Tables S2 and S3.

Data analysis

Statistical analysis was performed with the SPSS 18.0 (IBM, USA) and Origin 9.0 (OriginLab, USA). Independent sample *t* test was used to assess the possibility of significant differences. Any difference where $p > 0.05$ was not considered statistically significant. Spearman rank correlations were used to assess the association between N_2O emission and microbial parameters, together with biochemical parameters, and $p < 0.05$ was considered as significant. Network visualization was conducted using Gephi (<http://gephi.github.io/>). Redundancy analysis (RDA) concerning the relationship between evolution of nitrogen transformation functional genes and relevant environmental variables was conducted using Canoco 5.0 (Microcomputer Power, USA).

Results

Nitrogen transformation

As shown in Table 1, total nitrogen (TN) concentration increased and C/N ratio decreased in sludge bio-drying because of the concentrated effect caused by water loss and mineralization. The final TN content of pile I was much higher than that of pile II due to the higher concentrating effect of pile I caused by the better sludge bio-drying. The inorganic nitrogen forms, $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$, changed significantly during sludge bio-drying.

$\text{NH}_4^+\text{-N}$ accounting for most of the inorganic nitrogen was kept at a high level and led to an increase in pH during the thermophilic phase (days 1–5) of sludge bio-drying (Table 1). Meanwhile, the microbial activity of pile I was more intense as revealed by higher pile temperature (Fig. S2) which resulted in more DOC and proteins released, and thus $\text{NH}_4^+\text{-N}$ concentration, accumulated NH_3 emission, and pH in pile I were all higher than those of pile II at this stage (Table 1; Fig. 2a). These results were caused by low aeration which prolonged ammonification period and resulted in higher

Table 1 Chemical analysis of solid samples and N₂O emissions in each stage during sludge bio-drying

Piles	Sampling days	NO ² emission (g m ⁻²)	Chemical parameters									
			pH	NH ₄ ⁺ -N (g kg ⁻¹ DW)	NO ₃ ⁻ -N (mg kg ⁻¹ DW)	NO ₂ ⁻ -N (mg kg ⁻¹ DW)	TN ^a (g kg ⁻¹ DW)	C/N ratio	DOC (g L ⁻¹)	Porteins ^a (mg L ⁻¹)	Polysaccharide ^a (mg L ⁻¹)	
Pile I	Stage I	1	7.05 ± 0.12	1.63 ± 0.22	0.37 ± 0.15	1.06 ± 0.32	23.19 ± 0.72	9.19	1.05 ± 0.05	1.19 ± 0.42	0.63 ± 0.22	
		3	7.84 ± 0.04	2.51 ± 0.32	0.63 ± 0.16	0.34 ± 0.22	23.71 ± 0.53	8.45	1.41 ± 0.06	1.23 ± 0.42	0.81 ± 0.15	
		5	7.12 ± 0.06	1.43 ± 0.25	0.70 ± 0.22	54.69 ± 9.12	23.56 ± 0.62	7.84	1.05 ± 0.08	0.62 ± 0.22	0.53 ± 0.18	
		7	6.87 ± 0.08	0.44 ± 0.13	0.23 ± 0.09	32.29 ± 8.13	24.49 ± 1.10	8.13	1.09 ± 0.03	0.56 ± 0.18	0.53 ± 0.21	
	Stage II	11	6.64 ± 0.15	0.41 ± 0.05	1.87 ± 0.42	100.31 ± 20.25	24.54 ± 0.92	7.57	0.95 ± 0.04	0.50 ± 0.22	0.49 ± 0.13	
		14	6.74 ± 0.04	0.41 ± 0.12	2.46 ± 0.64	223.17 ± 35.42	22.18 ± 1.34	7.87	0.54 ± 0.02	0.45 ± 0.14	0.47 ± 0.10	
	Stage III	17	6.89 ± 0.05	0.38 ± 0.14	2.64 ± 0.55	125.13 ± 26.63	25.13 ± 0.82	7.76	0.76 ± 0.05	0.41 ± 0.16	0.40 ± 0.12	
		21	6.43 ± 0.07	0.43 ± 0.08	3.08 ± 0.42	122.88 ± 27.52	25.17 ± 1.51	7.91	0.94 ± 0.04	0.40 ± 0.15	0.41 ± 0.13	
	Pile II	Step I	1	6.89 ± 0.08	1.87 ± 0.56	0.41 ± 0.16	0.43 ± 0.08	23.85 ± 0.54	8.39	1.17 ± 0.02	1.05 ± 0.52	0.69 ± 0.20
		3	7.47 ± 0.09	2.05 ± 0.34	0.45 ± 0.08	0.34 ± 0.04	24.36 ± 0.62	7.69	1.10 ± 0.01	0.68 ± 0.22	0.65 ± 0.21	
	5	7.21 ± 0.10	2.36 ± 0.25	0.62 ± 0.22	0.28 ± 0.06	24.92 ± 0.24	7.69	1.13 ± 0.03	0.96 ± 0.18	0.67 ± 0.15		
	7	7.06 ± 0.11	0.44 ± 0.14	0.23 ± 0.13	19.84 ± 3.14	23.58 ± 1.12	8.35	0.85 ± 0.02	0.43 ± 0.14	0.43 ± 0.11		
	Step II	11	6.77 ± 0.09	0.45 ± 0.12	0.76 ± 0.22	88.53 ± 15.31	25.72 ± 1.03	7.97	1.01 ± 0.04	0.55 ± 0.15	0.56 ± 0.22	
	14	6.64 ± 0.10	0.44 ± 0.13	1.22 ± 0.18	168.66 ± 22.35	25.32 ± 1.25	7.96	0.94 ± 0.08	0.49 ± 0.13	0.48 ± 0.11		
	Step III	17	6.56 ± 0.12	0.41 ± 0.07	2.23 ± 0.42	232.78 ± 28.47	24.68 ± 0.85	7.39	1.00 ± 0.05	0.49 ± 0.12	0.53 ± 0.13	
	21	6.32 ± 0.15	0.33 ± 0.04	2.52 ± 0.33	288.51 ± 23.35	24.40 ± 1.22	7.41	0.83 ± 0.05	0.43 ± 0.10	0.46 ± 0.10		

^a Data from previous study (Zhang et al. 2015b)

TN total nitrogen, DW dry weight

Fig. 2 Evolution of NH_3 and N_2O flux and accumulated emission during sludge bio-drying

accumulated NH_3 volatilization in pile II. It is widely accepted that very little nitrification occurs at high pile temperature (Roig et al. 2001), and this was also demonstrated by the little change of NO_2^- -N and NO_3^- -N at the startup stage in this study.

As sludge bio-drying progressed, easily degraded organic matters have been depleted. Nitrification occurred when the pile temperature fell, and a high degree of nitrification was observed in both piles I and II after the thermophilic stage, e.g., significantly increase of NO_2^- -N and NO_3^- -N concentrations and significantly reduction of NH_4^+ -N concentration (Table 1). Similar to aerobic composting, the highest concentrations of NO_3^- -N in both piles occurred at the end of sludge bio-drying, but NO_3^- -N concentration of pile I were much higher than that of pile II (Table 1). Correspondingly, pH decreased along with nitrification due to decrease of NH_4^+ -N concentration.

Profile of N_2O emission

Corresponding to our previous study that sludge bio-drying should be divided into three stages based on the evolution of microbial community (Zhang et al. 2015b), and the profile of N_2O emission can also be divided into three stages accordingly. Slightly lower accumulated N_2O emission throughout the sludge bio-drying was observed in pile I (3.32 g m^{-2}) in comparison with pile II (3.42 g m^{-2}) (Fig. 2b), but N_2O emission showed different characteristics in different stages. At stage I (areas A

and B, days 1–5), the N_2O emission rates decreased gradually for both piles. However, the N_2O flux of pile I was significantly higher than that of pile II especially on day 3, which led to much higher accumulated N_2O emission of pile I (1.07 g m^{-2} , 31.9% of the total) than pile II (0.37 g m^{-2} , 10.5% of the total) as listed in Table 1. This may be associated with aeration strategy of the pile temperature feedback control model, in which aeration was started if pile temperature was above the set temperature. The pile temperature of pile I was always higher than the set temperature at stage I, which led to significantly higher aeration rate, conversely, the pile temperature of pile II was lower than the set value (Fig. S2). Besides, the highest N_2O emission detected during the thermophilic stage was confirmed previously for sludge composting process (Maulini-Duran et al. 2013).

At stage II (areas C and D, days 5–14), N_2O flux reached its peak value on day 11 and then decreased dramatically in both piles. The N_2O emission mainly happened at this stage during sludge bio-drying. Not only the peak N_2O flux of pile II ($15.49 \text{ mg m}^{-2} \text{ h}^{-1}$) was much higher than that of pile I ($12.62 \text{ mg m}^{-2} \text{ h}^{-1}$) but also the accumulated N_2O emission of pile II (1.99 mg m^{-2} , 58.2% of the total) at this stage was much higher than that of pile I (1.70 mg m^{-2} , 53.3% of the total). At stage III (areas E and F, days 14–21), the N_2O flux of pile II (4.5 – $8.9 \text{ mg m}^{-2} \text{ h}^{-1}$) was much higher than pile I (2.87 – $3.32 \text{ mg m}^{-2} \text{ h}^{-1}$), and the accumulation N_2O emission was 0.55 g m^{-2} (16.6% of the total) and 1.07 g m^{-2} (31.3% of the total) for piles I and II, respectively (Fig. 2b).

Changes of nitrification and denitrification genes

Previous studies have demonstrated that N_2O emission had a significant correlation with environmental oxygen supply (He et al. 2001). Thus, it is assumed that aeration strategy during sludge bio-drying would alter the bacterial gene abundance for key nitrogen transformation functional enzymes, which correlate to changes in N_2O emission. As shown in Figs. 3 and 4, the gene copies and abundance changed a lot throughout the sludge bio-drying. Except for *amoA* and *hao*, the gene copies of all the other genes increased to their peak at their corresponding highest pile temperature (stage I), and then decreased dramatically (stages II and III) during sludge bio-drying. Statistical analysis showed that except on days 5 and 21, the gene copies of *amoA* ($\text{NH}_4^+\text{-N} \rightarrow \text{NH}_2\text{OH}$) of pile II was significantly higher than that of pile I, and increased throughout the sludge bio-drying from 8.04×10^8 to 8.16×10^{10} copies g^{-1} DW. The gene copies of *hao* ($\text{NH}_2\text{OH} \rightarrow \text{NO}_2^-\text{-N}$) decreased significantly during the thermophilic phases while increased gradually afterwards along with the enhancement of aeration from 3.33×10^5 to 3.84×10^6 copies g^{-1} DW. However, the gene copies of *nrrA* ($\text{NO}_2^-\text{-N} \rightarrow \text{NO}_3^-\text{-N}$) showed the similar profile to denitrification genes and ranged from 2.04×10^3 to 4.13×10^5 copies g^{-1} DW. While for denitrification genes, *narG* ($\text{NO}_3^-\text{-N} \rightarrow \text{NO}_2^-\text{-N}$) was the highest throughout the sludge bio-drying (Figs. 3 and 4) with the gene copies ranging from 2.47×10^9 to 5.06×10^{10} copies g^{-1} DW. All the denitrification genes reached maximum at stage I, gradually decreased along with time at stage II and changed little at stage III. This may be due to the need of anaerobic condition for the ongoing of denitrification, while aerobic conditions did not avail the process of denitrification. The increasing aeration as sludge bio-drying progressed as shown in Table S1 and the decreasing microbial activity reflected from the evolution of temperature shown in Fig. S2 led to the transition of anaerobic condition to the aerobic conditions. Except on day 1, almost all denitrification genes and *nrrA* in pile II was significantly higher than those in pile I. This may be due to earlier peak pile temperature of pile I on day 1. Although the *nirK* and *nirS* gene ($\text{NO}_2^-\text{-N} \rightarrow \text{NO}$) both can reduce nitrite, this study revealed that the gene copies of *nirS* was approximately 2 logs higher than that of *nirK*, and this dominance of *nirS* over *nirK* was consistent throughout the sludge bio-drying. This result was congruent with previous studies reporting that the *nirS* gene was environmentally more abundant than *nirK* gene (Kandeler et al. 2006; Wang et al. 2013). The gene copies of *nosZ* gene ($\text{N}_2\text{O} \rightarrow \text{N}_2$)—the only sink for N_2O ranged from 1.72×10^9 to 2.77×10^{10} copies g^{-1} DW, while the gene copies of *norB* ($\text{NO} \rightarrow \text{N}_2\text{O}$) ranged from 3.55×10^6 to 1.04×10^8 copies g^{-1} DW.

Discussion

Effects of aeration strategy on biochemical parameters corresponding N_2O emission

Little changes of $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations at stage I indicated that N_2O emission may be completed by traditional denitrification. Also, sufficient carbon sources (higher DOC, polysaccharide, and proteins) and low aeration strategy at this stage availed the denitrification of heterotrophic denitrifiers. At stage II, heterotrophic denitrifiers were severely inhibited due to higher aeration rates as shown in Table S1. However, the intense ongoing of the nitrification indicated by significant increase of concentrations of $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ showed that nitrifiers may contribute to the N_2O emission at stage II. While there are mainly two ways of N_2O emission for nitrifiers: nitrifier denitrification by ammonia-oxidizing bacteria (AOB) (Wunderlin et al. 2012) and biological NH_2OH oxidation (Peng et al. 2014; Sutka et al. 2006). However, N_2O production from the latter is not expected to be dominant as the assumed much lower NH_2OH concentration in sludge (Wunderlin et al. 2012) and being an energy-generating step for NH_2OH oxidation to N_2O (Casciotti et al. 2003). Thus, nitrifier denitrification by AOB might be one of the primary N_2O production pathways at stage II, and the environmental conditions (aerobic conditions, low available carbon sources, and the presence of nitrite) for nitrifier denitrification here were in accordance with previous study (Wunderlin et al. 2012). Meanwhile, the aeration rate at stage II for pile I was lower than pile II, and higher aeration rate availed nitrifiers, and thus, N_2O flux and accumulated emission were both much higher for pile II than pile I.

As sludge bio-drying progressed, piles entered into stage III (areas E and F) in which aeration rates for both piles were very high. At this stage, it is not a microbial process for sludge bio-drying, but rather a physical one (Zhang et al. 2015b), which led to severe inhibition of microbial process including nitrifier denitrification due to high aeration and low MC. Lower $\text{NO}_2^-\text{-N}$ accumulation for pile I due to higher aeration rates maybe the reason for the lower N_2O emission at stage III.

Effects of aeration strategy on nitrogen transformation functional genes corresponding to N_2O emission

From the point of view of nitrogen transformation functional genes, we concluded the same results as the changes of biochemical parameters suggested on the profile of N_2O emission during sludge bio-drying. At stage I, the abundance of *amoA* and *hao* changed little, while gene copies and abundance of other functional genes reached their maximum (Figs. 3 and 4). This indicated that denitrification by heterotrophic denitrifiers contributed the N_2O emission, and the accumulated N_2O emission of pile I higher than pile II may attribute to lower

Fig. 3 Evolutional changes of gene copies per DW for 16S rRNA, *amoA*, *hao*, *nxrA*, *narG*, *nirK*, *nirS*, *norB*, and *nosZ* on days 1, 3, 5, 7, 11, 14, 17, and 21 during sludge bio-drying for piles I and II. Error bars indicate standard deviations of triplicate qPCR reactions. * $p < 0.05$, different significant rates

gene copies of the only sink for N_2O (*nosZ*) on day 3. At stage II, the gene copies of *amoA* increased to its maximum, while gene copies of denitrification functional genes and *nxrA* decreased significantly, which indicated that nitrifiers (especially AOB) contributed to the N_2O emission at this stage. Interestingly, the gene copies and abundance of *hao* decreased to the minimum in this stage (Figs. 3 and 4), which indicated that amounts of NH_2OH accumulated and N_2O emission due to NH_2OH oxidation also existed at stage II, especially when

the maximum N_2O emission (Fig. 2b) happened at the time of the minimum gene copies of *hao* gene (Figs. 3 and 4). As revealed by Fig. 1, *Nir* and *Nos* genes were the same genes used by nitrifiers denitrification. It was generally accepted that the activity of *norB* gene is approximately 10 times higher than that of *nirS/nirK*, thus ensuring little accumulation of the toxic NO (Otte et al. 1996). Thus, *nirS* and *nirK* gene instead of *norB* gene can be recognized as N_2O producing bacteria, while *nosZ* as N_2O consuming bacteria (Wang et al.

Fig. 4 Heatmap of changes of the abundance of the relevant nitrogen transformation functional genes quantified in this study. Values plotted are the natural log₂ transformed of the abundance of each gene

2013). The order of enzymes relevant to nitrogen transformation function sensitive to oxygen was as follows: *nosZ* > *narG* > *nirS/nirK* (Lu et al. 2014). As the most sensitive enzyme to oxygen, *nosZ* gene resulted in transient N₂O emission after significantly increased aeration rate at stage II. This was also supported by the result of Lu and Chandran (2010) who concluded that transient exposure to oxygen led to increased N₂O emission in a denitrifying sequencing batch reactors. As the increasing aeration rate, *nosZ*-N₂O consuming bacteria would be severely inhibited, and thus, N₂O emission mainly attributed to N₂O producing bacteria reflected by gene copies of *nirS* in this study due to significantly higher gene copies over *nirK* throughout the sludge bio-drying in both piles. This was further confirmed by the significantly positive spearman correlation ($p = 0.009$) between N₂O emission and *nirS*/16S rRNA (Fig. 4). Meanwhile, the gene copies of *amoA* and *nirS* were significantly higher for pile II than pile I, which led to higher N₂O emission for pile II at stage II. As sludge bio-drying entered into areas E and F, aeration rate increased further, and N₂O emission increased to a maximum and decreased with additional oxygen which may be high enough to inhibit the enzymes of *narG* and *nirS/nirK*. The gene copies of *hao* increased gradually at stage III, and this may be due to the fact that higher aeration build an aerobic condition and *hao* recovered upon recovery to aeration condition while decreased during the anoxic conditions as suggested previously (Yu et al. 2010). However, the gene copies of *amoA* and *nirS* were still significantly higher for pile II than pile I, which also led to higher N₂O emission for pile II at stage III.

Statistical correlations indicating the mechanism of N₂O emission in sludge bio-drying

Network analysis confirmed the interaction and ecological association among the nitrogen transformation microbial communities (Fig. 5). All experimental data were used to determine spearman correlations to compare the N₂O flux with biochemical and environmental parameters and microbial gene abundance. Statistically significant correlations were found between the NO₃⁻-N and NO₂⁻-N concentrations and pH, *amoA* indicated that nitrification was responsible for the falling pH values. Moisture content (MC) and pile temperature were the most important environmental parameters shown in Fig. 5. Also, RDA analysis showed C/N, temperature, organic matters, and MC contributed significantly to the evolution of nitrogen transformation functional genes, while NO₂⁻-N and NO₃⁻-N have significant correlation with the gene copies and abundance of *amoA* (Fig. 6). NH₃ emission and inorganic nitrogen have significant correlations with many microbial and biochemical parameters, such as pH, MC, temperature, *amoA*, *nirS*, and *nosZ*.

Interestingly, we observed a significant correlation between the N₂O flux and *nirS*/16S rRNA ($p = 0.009$, positive), *nosZ*/*(nirS + nirK)* ($p = 0.022$, negative), and *nosZ/nirS* ($p = 0.047$, negative). Numerous studies have focused on clarifying the correlation between denitrification functional genes and N₂O emission. Regan et al. (2011) found the predominant nitrite reductase gene was *nirK* rather than *nirS* and demonstrated that high N₂O emission under elevated CO₂ correlated with lower *nosZ/nirK* ratios. Philippot et al. (2011) stated that N₂O production correlated with the *nosZ*/16S rRNA ratio and *nosZ/narG* ratio. A strong correlation between N₂O emission and *nirS* gene abundance minus *nosZ* gene abundance has been reported by Morales et al. (2010). Wang et al. (2013) particularly suggested that the abundances of *nirK* and *nosZ* genes were likely to play a dominant role in controlling N₂O emission. Although not universally compatible with all types of environmental conditions, the relationship between N₂O emission and the abundance of denitrification functional genes were generally observed. In the current study, no correlation was observed between N₂O emission and *nirS* – *nosZ* gene abundance or the *nosZ*, *nirK*, and *nosZ/narG*. The data in the current study particularly suggested that *nirS*/16S rRNA, *nosZ/(nirK + nirS)*, and *nosZ/nirS* ratios were likely to play a dominant role in N₂O emission during sludge bio-drying. These correlations were also supported by previous studies (Harter et al. 2014). Meanwhile, although targeted denitrifying gene abundances were strongly linked to measured N₂O flux in this study, that this denitrification was conducted by AOB should be emphasized with the high aeration rates used in this study, and the sludge bio-drying was kept predominantly aerobic at stages II and III.

However, anoxic or anaerobic microsites might still exist inside the waste particles similarly to the coexistence of aerobic and anaerobic microenvironment in aerobic soils (He et al. 2001); denitrification conducted by heterotrophic denitrifiers in these microsites might contribute to NO₂⁻-N reduction. If this is the case, N₂O production should be proportional to the available carbon. This was confirmed by the significantly positive correlations of gene *nirS* and *nosZ* with DOC, proteins and polysaccharide, while significantly negative correlation of gene *amoA* with DOC, proteins, and polysaccharide. However, most of the N₂O emission was produced after the depletion of easily available organic carbon in our study (Table 1; Fig. 2), a result inconsistent with the active metabolism of heterotrophic denitrifiers. The sequential appearance of NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N peaks might prove the existence of nitrifiers, and nitrifier denitrification appeared to be the predominant mechanism for N₂O production during sludge bio-drying. This was also demonstrated by He et al. (2001) who found that nitrifier denitrification appeared to be the predominant mechanism for N₂O emission in an aerated composting of organic waste.

Fig. 5 The network analysis revealing the correlation among microbial and biochemical parameters and N₂O emission. The nodes were colored according to modularity class. A connection represents a significant correlation ($p < 0.05$). The size of each node represents the degree of connections. Green edge presents positive correlation, while red edge indicates negative correlation. MC moisture content

In a word, this research determined which kind of microbes largely contributes to N₂O emission during sludge bio-drying. The different contributors to N₂O emission at different stages were clarified, and both nitrifier denitrification and NH₂OH accumulation by AOB were the primary contributors to the N₂O emission during sludge bio-drying. Results confirmed that aeration strategy did affect N₂O emission and nitrogen

transformation functional genes, and regulating the aeration strategy has the potential to lessen N₂O emission. Although the aeration strategy at stages II and III was indeed improved for pile I, the aeration strategy at stage I needs further optimization for N₂O emission reduction, i.e., reducing the aeration time on area A just like pile II may be a good way for the further reduction of N₂O emission for pile I especially at stage I.

Fig. 6 Redundancy analysis (RDA) results of the relationship between evolution of nitrogen transformation functional genes and relevant environmental variables. The blue lines and red lines represented the species variables and environmental variables, respectively

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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