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Roles of *nxrA*-like oxidizers and *nirS*-like reducers in nitrite conversion during swine manure composting

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ABSTRACT

Nitrite has a key role in nitrogen conversion during composting. In this study, the dynamic changes in the NO_2^- contents, abundances of *nirS* and *nxrA*, and the bacteria that harbored these genes were determined during composting. NO_2^- accumulated during the initial composting stage. The *nirS* gene was abundant throughout composting, whereas the *nxrA* gene was only abundant in the late composting phases. *Ralstonia* sp. and *Thauera* sp. were the dominant denitrifiers that harbored *nirS*, and *Nitrobacter winogradskyi Nb-255* was the dominant nitrifier that harbored *nxrA*. Structural equation modeling showed that NO_2^- was mainly reduced by *nirS* in the early phases, and oxidized by *nxrA* in the late phases, but especially in the maturity phase. Network analysis showed that the dominant bacteria harboring *nirS* and *nxrA* were hubs in the modules related to the reduction and oxidation of NO_2^-, and they had competitive relationships during the cooling and maturity phases.

1. Introduction

In China, about 3.8 billion tons of livestock and poultry manure are produced each year (Tian, 2012), among which swine manure accounts for about 20.4%. Swine manure contains many nutrients and organic matter, but it also contains many unstable organic substances that are harmful to the soil, plants, and even the environment, thereby posing a global environmental challenge (Posmanik et al., 2014). Composting is an economical and effective technique for utilizing organic solid waste (Gajalakshmi and Abbasi, 2008). In particular, composting is considered to improve land and it is important for providing a stable product containing abundant nutrients that are readily available to plants (Zeng et al., 2011).

The biochemical conversion of various organic substances occurs during the composting process, where the conversion of elemental nitrogen plays an important role in synthesis and oxidation by microorganisms (Yin et al., 2016). Some organic nitrogen compounds in the raw materials are mineralized during composting and humic substances are synthesized. However, nitrogen is mainly lost as ammonia, nitrous oxide, and nitrogen monoxide. Cáceres et al. (2018) reported that about 46.8–77.4% of the nitrogen in composting manure is lost as ammonia, and recent studies have shown that nitrification plays an important role in the fixation of nitrogen, where it can ultimately convert ammonia into nitrate to reduce the release of ammonia (Cáceres et al., 2018; Maeda et al., 2011). Ammonia-oxidizing bacteria/archaea can oxidize ammonia into NO_2^- (Yin et al., 2016), which is a rate-limiting step in nitrification, whereas NO_2^- is further oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB) (Li et al., 2016a). In recent years, many studies have investigated the changes in the NOB community during composting as well as variations in the related genes (Cáceres et al., 2018; Li et al., 2016a; Maeda et al., 2011). *Nitrobacter* species are considered to have key roles in nitrogen cycling by converting nitrite into nitrate in the terrestrial ecosystem, and some studies have used *nxrA* as a key gene to detect NOB in compost. For instance, Li et al. (2016a) found that *nxrA* had a negative correlation with temperature and it was inhibited by amendment with flue gas desulphurization gypsum during the mature phase. Zhang et al. (2017a) found that the abundance of *nxrA* increased significantly in the maturation phase, which suggests

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that nitrification occurs mainly in the late stage of composting.

Studies have shown that denitrification can also occur during composting (Li et al., 2016b; Maeda et al., 2017; Zhang et al., 2015), where nitrite-reducing bacteria (NRB) can reduce NO_2^- to nitric oxide, which further increases the loss of nitrogen from compost and harms the atmosphere. In addition, Fukumoto et al. (2006) found that inoculating compost with NOB reduced the nitrous oxide emissions and the accumulation of nitrite. Nitrite can be utilized as a substrate by NOB and NRB during nitrogen conversion (Li et al., 2016a). Therefore, changes in the NOB communities can affect the denitrifying microorganisms present in compost, where the most important are the NRB that carry the *nirS* and *nirK* genes. Previous studies have shown that *nirS* is widely distributed in the environment (Zhang et al., 2017b) and it plays an important role in reducing nitrite (Miao et al., 2015; Li et al., 2016a). Zhang et al. (2015) found that *nirS* is more important during the early phases of composting (especially in the high temperature phase) than the *nirK* gene, and it is correlated with the concentration of NO_3^- according to an inverse model.

Previous studies have shown that nitrite can accumulate in compost (Fukumoto and Inubushi, 2009; Wang et al., 2016, 2018; Wu et al., 2018a), and that both *nxrA* and *nirS* are actively involved in nitrite oxidation and reduction during the composting process. However, the roles of NOB and NRB during composting as well as their relative contributions to nitrite conversion in different composting phases remain unclear. The present study investigated the role of the *nxrA* and *nirS* genes to nitrite conversion during different phases in the composting process. Quantitative PCR (qPCR) and high throughput sequencing were conducted to detect the abundances of the *nxrA* and *nirS* genes during composting, as well as the diversity and structure of the bacterial community that harbored these genes. The results obtained in this study may facilitate further research into improving the quality of swine manure compost.

2. Materials and methods

2.1. Experimental setup

Fresh swine manure was collected from a pig farm (longitude 107.58°E, latitude 34.18°N) in Yangling, Shaanxi, China, and it was allowed to dry naturally in the air until the moisture content was less than 30%. Wheat straw was collected from an experimental field at Northwest Agriculture and Forestry University (longitude 108.08°E, latitude 34.29°N), China, and it was chopped into pieces measuring less than 1 cm in length. The swine manure had an organic nitrogen content of 26 g kg^{-1}, organic carbon content of 380.2 g kg^{-1}, and pH of 7.6. The wheat straw had an organic nitrogen content of 6.5 g kg^{-1} and an organic carbon content of 496.3 g kg^{-1}.

The compost reactors comprised 15 identical 500-mL plastic containers (including 5 samples, and 3 repetitions per sample), and each reactor contained a mixture of 100 g dry swine manure and 50 g dry wheat straw (Yin et al., 2017), and the moisture content was adjusted to 55%. The entire composting process was conducted in an incubator and the temperature of the incubator was artificially controlled according to the optimal composting conditions in the following four phases: (1) the compost temperature ranged from 20 °C to 55 °C in the first five days (mesophilic phase); (2) the temperature was controlled to above 50 °C from day 6 until day 16, where the temperature was 55 °C from days 6–10, 55–50 °C from days 11–13, and 50 °C from days 14–16 (thermophilic phase); (3) the composting temperature decreased from 50 °C to 40 °C during days 17–21 (cooling phase); and (4) the final temperature decreased to 20 °C during days 22–35 (maturity phase), as described previously (Li et al., 2014). The compost reactors were turned fully and mixed every two days during the composting process.

2.2. Sampling and chemical analyses

The compost was sampled in triplicate (samples from three plastic reactors were mixed until they were homogeneous) on days 2, 7, 14, 21, and 35, and the sample was divided into two parts. The first part was used to determine the physical and chemical properties of the compost sample. The second part was freeze dried using a vacuum freeze dryer (Songyuan, China), milled to a diameter of 1 mm in an ultra-centrifugal mill (Retsch Z200, Germany), and stored at –80 °C. The moisture contents of the fresh samples were measured based on the weight lost by drying at 105 °C for 24 h. The pH and electrical conductivity (EC) values were determined using a fresh compost sample mixed with deionized water 1:10 (w/w), which was mechanically shaken at 200 rpm for 30 min to extract the suspension, before measuring with a pH meter (CA, USA) and conductivity electrode (DDS-11A, Shanghai, China), respectively. NH_4^+, NO_2^-, and NO_3^- measurements were obtained using fresh compost samples mixed with 2 mol L^{-1} KCl at 1:50 (w/w), which were then mechanically shaken at 150 rpm for 60 min to extract the suspension and analyzed colorimetrically by flow injection analysis (Systea, Italy).

2.3. DNA extraction and qPCR detection of nxrA and nirS genes

DNA was extracted from freeze-dried compost samples using a Fast DNA Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. The copy numbers of the *nxrA* and *nirS* genes were quantified in triplicate by qPCR (CFX connect™ Real-Time PCR, BioRad) for each sample with an *nxrA* and *nirS* gene primer set comprising: nxrA1F/nxrA1R (Li et al., 2016a), and cd3af/R3cd (Chen et al., 2014), respectively. The qPCR reaction conditions were as follows: (1) 95 °C for 10 min and (2) 40 cycles at 95 °C for 10 s, 59 °C (*nxrA*) or 58 °C (*nirS*) for 30 s, and 72 °C for 32 s. Melting point curve analysis was conducted at the end of the reactions in order to verify the specificity of the amplicons.

2.4. High-throughput sequencing analysis of nxrA and nirS genes

High-throughput sequencing was conducted for the *nxrA* and *nirS* genes using the Illumina platform, where the primers comprising nxrA1F/nxrA1R and cd3aF/R3cd were employed for the PCR amplification of *nxrA* and *nirS*, respectively. Each 5′-end of the forward primer had a unique barcode to distinguish the sample.

After sorting the sample sequences based on their barcodes, the barcodes and primer sequences were deleted. Raw data analysis was conducted according to the procedures described by Bertagnolli et al. (2016) and Zhang et al. (2019), and sequences with translated proteins that did not match the *nxrA* and *nirS* protein sequences or that contained termination codons were discarded. The remaining high-quality sequences were grouped into operational taxonomic units (OTUs). Representative sequences of *nxrA* and *nirS* in the OTUs were subjected to searches with the BLAST algorithm via GenBank in the NCBI database (http://blast.ncbi.nlm.nih. gov/Blast.cgi). Sequences were edited, aligned, and translated using MEGA 5.0, and their phylogeny was constructed based on the neighbor-joining method, where bootstrap tests were performed with 1000 replicates.

2.5. Statistical analyses

All of the statistical analyses were performed using SPSS 19.0 (SPSS Inc., USA). Significant differences in the abundances of *nxrA* and *nirS* (at $P < 0.05$) between samples collected on different days were detected using one-way analysis of variance and the least significant difference test. A structural equation modeling (SEM) was constructed using IBM SPSS AMOS 22.0 in order to study the roles of the *nxrA* and *nirS* genes, and the compositions of the communities that harbored *nxrA* and *nirS* in nitrite conversion. Before SEM analysis of the organisms that

Table 1
Changes in the physicochemical parameters during the composting of swine manure.

Compost Sample	Day-2	Day-7	Day-14	Day-21	Day-35
pH	8.72 ± 0.03	9.14 ± 0.00	8.38 ± 0.02	8.21 ± 0.01	8.51 ± 0.05
EC (mS cm^{-1})	4.71 ± 0.01	4.01 ± 0.01	3.04 ± 0.01	2.48 ± 0.01	1.98 ± 0.02
NH_4^+ (mg kg^{-1})	2187.95 ± 76.17	2386.95 ± 92.81	1440.44 ± 14.43	123.34 ± 3.51	201.00 ± 3.46
NO_3^- (mg kg^{-1})	221.69 ± 7.77	188.57 ± 23.93	115.31 ± 6.86	97.80 ± 2.34	219.66 ± 5.94
NO_2^- (mg kg^{-1})	35.21 ± 2.18	1.29 ± 0.08	–	–	–

harbored *nxrA* and *nirS*, principal component analysis (PCA) was conducted with SPSS 19.0 (SPSS Inc., USA), where PC1 represented the community composition (data not shown) (Zhang et al., 2018). The heatmaps graph was conducted using R (Version 3.3.1). Network analysis with Spearman's rank analysis was performed using the abundances of *nxrA* and *nirS*, the communities that harbored *nxrA* and *nirS* (based on OTU), and NO_2^- production with the Gephi platform (0.9.2).

3. Results and discussion

3.1. Changes in physicochemical parameters during swine manure composting

The pH increased from 8.72 to 9.14 in the first 7 days, before decreasing to 8.38 during days 7–14, and it then remained constant until the end of composting (Table 1). A previous study found that as the temperature increased during composting, the microbial activity also increased rapidly (with the degradation of organic acids and the release of ammonia), which led to an increase in the pH during the initial phase of composting (Bernal et al., 2009). By contrast, the decrease in the pH during the later phase was probably a consequence of the synthesis of phenolic compounds or the nitrifying process (Yin et al., 2019). The EC decreased during composting and the EC was 1.98 mS cm^{-1} on day 35 (Table 1), which indicates that the compost would not cause any phytotoxicity after its application (Chan et al., 2016). The changes in the NH_4^+, NO_2^-, and NO_3^- concentrations indicated N-mineralization during composting, where the NH_4^+ concentration increased during the first 7 days, before decreasing and then remaining at about 201 mg kg^{-1} (Table 1). The release of NH_4^+ via ammonification coincided with the active degradation of organic matter during the thermophilic phase (Chan et al., 2016). The NO_3^- concentration peaked on day 2, decreased during days 7–21, and the increased until day 35 (Table 1). These changes may have been due to the intense activity of nitrifying bacteria during the maturity phase. Cáceres et al. (2018) also reported that ammonia-oxidizing archaea (AOA)/ammonia oxidizing bacteria (AOB) and NOB play key roles in nitrogen conversion during the later phases of composting. In the present study, the concentration of NO_2^- was lower throughout the composting process, with a peak when the NO_2^- concentration reached 35.21 mg kg^{-1} on day 2, but the concentration was below the detection limit after 7 days (Table 1), as also found by Wang et al. (2016).

3.2. Changes in abundances of bacterial nirS and nrxA genes during swine manure composting

The changes in the abundances of *nxrA* and *nirS* in this study are shown in Fig. 1. The *nirS* gene was abundant throughout the entire composting process, i.e., from 1.63×10^{10} to 4.65×10^{11} copies (Fig. 1a). In the mesophilic and initial thermophilic phases, the abundances of *nirS* were 95.7–96.5% and 86.1–88.7% higher, respectively, than those in the other phases. Li et al. (2016b) also found that denitrifying microorganisms can still maintain their metabolic activities under high temperature conditions, and the *nirS* gene was identified as a key indicator gene of denitrification during the accumulation of NO_2^-. However, the abundance of *nirS* gradually decreased throughout the composting process, which is consistent with the results obtained in

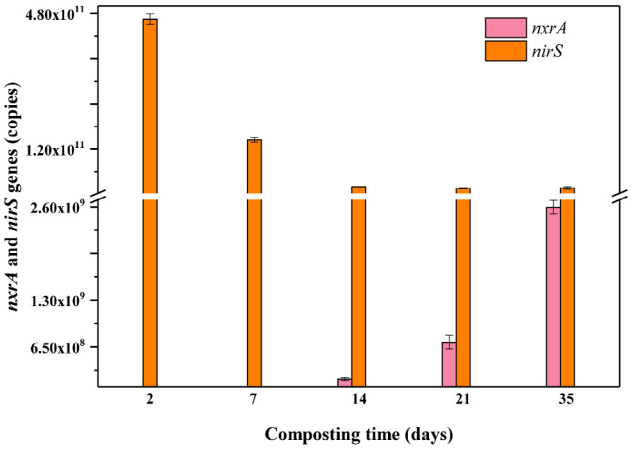

Fig. 1. Changes in abundances of the *nxrA* and *nirS* gene during swine manure composting.

a previous study (Maeda et al., 2017; Zhang et al., 2015). These findings indicate that communities harboring *nirS* played a role in denitrification during the early phases, and previous studies also found that NO and N_2O were produced as the concentrations of NO_2^- decreased during the early stage of composting (Chen et al., 2018; Pan et al., 2019; Wu et al., 2018a), which may have been related to the activities of communities that harbored *nirS* (Cui et al., 2019).

The *nxrA* gene is a marker of the oxidation of NO_2^- to NO_3^-, and the abundance of this gene varied greatly among the samples (Fig. 1b). The *nxrA* gene was undetectable during the first 14 days, possibly because the accumulation of NO_2^-, NH_4^+, and salt might have provided unfavorable conditions for the NOB community during this period. Fukumoto et al. (2006) reported that the accumulation of NO_2^- during composting delayed the growth of NOB, and Posmanik et al. (2014) also found that NOB were sensitive to NH_3. In addition, NOB are highly sensitive to salt (Cáceres et al., 2018) and a previous study showed that EC can reflect the salt content of compost (Lin, 2008). In the present study, the NO_2^- and NH_4^+ contents as well as the EC values were significantly higher in the first 7 days. The abundance of the *nxrA* gene gradually increased during the composting process, where the abundance on day 35 was 12.1 times higher compared with that on day 14, thereby suggesting that NOB were important during the later phase of composting. Zeng et al. (2012) also found that nitrification occurred mainly in the late composting period, and Li et al. (2016a) found that the *nxrA* gene was strongly associated with changes in the NO_3^- content during the compost maturity phase.

3.3. Structure of communities that harbored nirS and nxrA

The structures of the communities that harbored *nirS* and *nxrA* were characterized by investigating these genes using high-throughput sequencing. Sequencing yielded a total of 104,058 high-quality *nxrA* and 215,445 *nirS* gene sequences, and each sample generated 147–219 OTUs for *nirS* and 5–23 OTUs for *nxrA*. The community that harbored *nirS* was sensitive to temperature, and the Shannon index decreased after entering the high temperature stage, whereas it then gradually

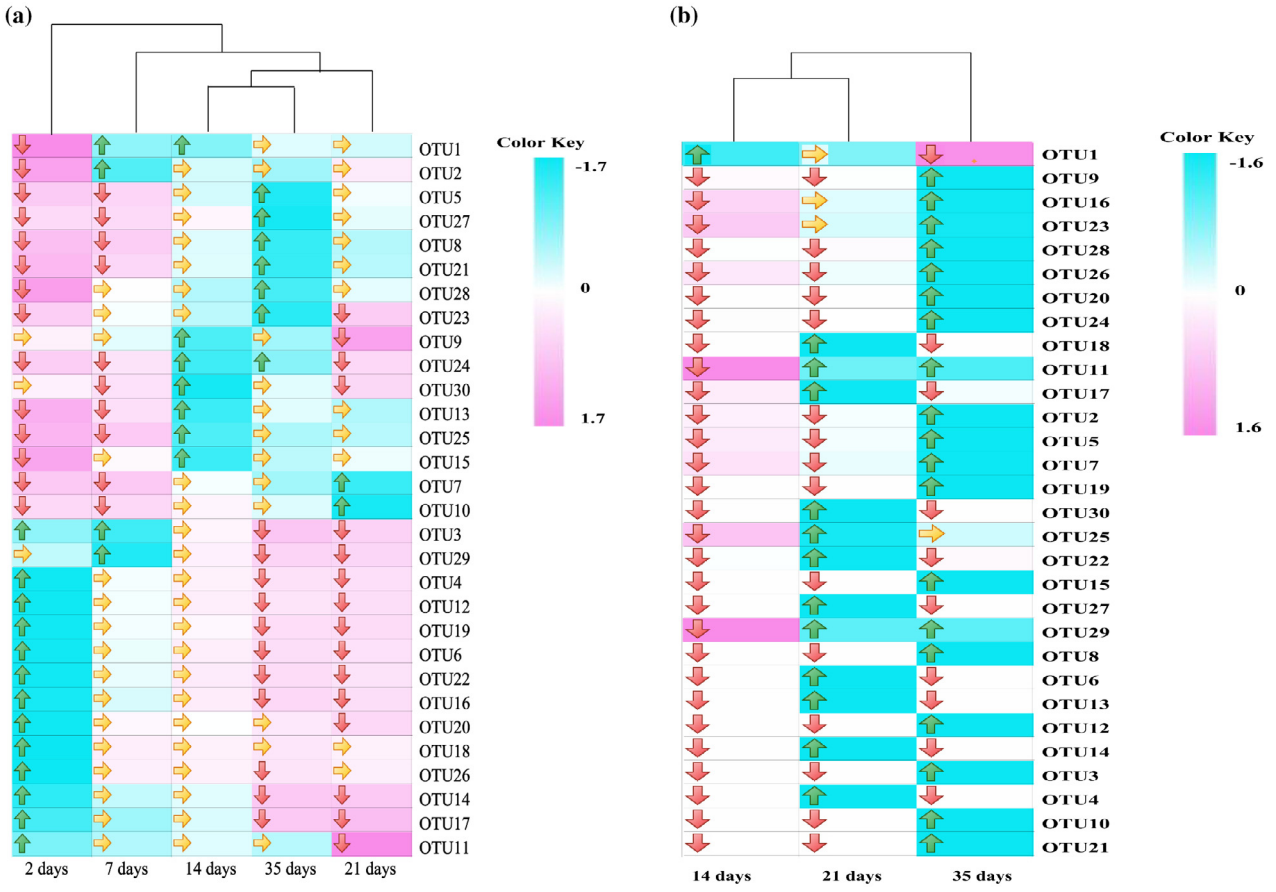

Fig. 2. Heatmaps showing the top 30 OTUs in the community that harbored *nirS* (a) and *nxrA* (b) during swine manure composting.

increased as the temperature decreased. However, the ACE and Chao indices gradually decreased throughout the composting process. The Shannon index determined for the community that harbored *nxrA* continued to increase from 0.08 on day 14 and reached 2.01 at the end of composting. Similar to the Shannon index, the ACE and Chao indices increased in the late composting period.

The heatmaps in Fig. 2 show the abundances of the top 30 OTUs for the communities that harbored *nirS* and *nxrA*. For the *nirS* gene, all of the compost samples (columns in heatmap) could be broadly separated into three clusters according to the composting phases: mesophilic (day 2), initial thermophilic (day 7), and other phases (Fig. 2a). OTU1 and OTU2 dominated the composting phases, where their abundances were 167.5–239.3% and 79.0–220.2% higher, respectively, in the other phases compared with the mesophilic phase. These findings indicate that a similar predominant community harbored *nirS* in all of the phases. Phylogenetic analysis showed that OTU1 and OTU2 were closely related to *Ralstonia* sp. UNPF19a and *Thauera* sp Q20-C (Fig. 3a), respectively, and similar findings were reported by Maeda et al. (2018). OTU3, OTU4, OTU6, OTU12, and OTU16 were dominant in the mesophilic phase, but the abundances decreased by 56.9–89.2%, 76.4–96.1%, 72.8–96.3%, 74.1–94.7%, and 64.5–97.1%, respectively, as the composting process continued. Thus, the decrease in the abundance of the *nirS* gene may have been related mainly to the microorganisms represented by these OTUs, which were closely related to *Azoarcus tolulyticus* strain *2FB6*. These microorganisms may be indigenous bacteria in feces and closely related to aerobic denitrifying bacteria (Song and Ward, 2003). These bacteria could have been unsuited to competition under the high temperature stress and low oxygen supply conditions during composting (Wang et al., 2015; Xi et al., 2015). In addition, OTU5, OTU7, OTU8, OTU10, and OTU27 were rare in the mesophilic and thermophilic phases, but they dominated in the later stage of composting, especially in the maturity phase. These OTUs were closely related to *Azoarcus* sp. *DN11*, *Cupriavidus* sp. *NC3H-95a*, and *Thauera* sp. *Q20-C*, and a previous study found that these bacteria may be the main *nirS*-harboring denitrifiers in organic fertilizer and manure, and they are dominant in the soil after the application of manure and organic fertilizer (Tao et al., 2018).

For the *nxrA* gene, the abundance data were grouped in the thermophilic and cooling phases, whereas the compost maturity phase was clearly separated from the other phases. OTU1 was dominant in the thermophilic and cooling phases, but its abundance decreased by 75.8% at the end of composting (Fig. 2b). OTU1 was closely related to *Nitrobacter winogradskyi Nb-255* (Fig. 3b). Thus, *Nitrobacter winogradskyi Nb-255* may have been unsuited to the environmental conditions on day 14, possibly because this type of NOB favors temperatures less than 50 °C. The decline in OTU1 may also have been related to the NO_2^- content, which it oxidizes as an energy source. Sayavedra-Soto et al. (2015) found that *Nitrobacter winogradskyi* can grow only on NO_2^- as a source of energy and nitrogen. The abundances of OTU2, OTU3, and OTU4 increased in the cooling phase, but they were dominant in the maturity phase. OTU2 was closely related to *Streptomyces* sp. *ADI95-16*, which may enter the soil and participate in the nitrification process. Han et al. (2017) found that *Streptomyces* spp. are very important NOB in the soil environment. By contrast, OTU15, OTU19, and OTU22 were present only in the maturity phase, and these OTUs were closely related to *Nitrosospira lacus strain APG3*. Jarvis et al. (2009) also detected *Nitrosospira* during the compost maturation phase. Previous studies demonstrated that *Nitrosospira* spp. are the most important nitrifying bacteria in composting (Maeda et al., 2011; Yamada et al., 2013).

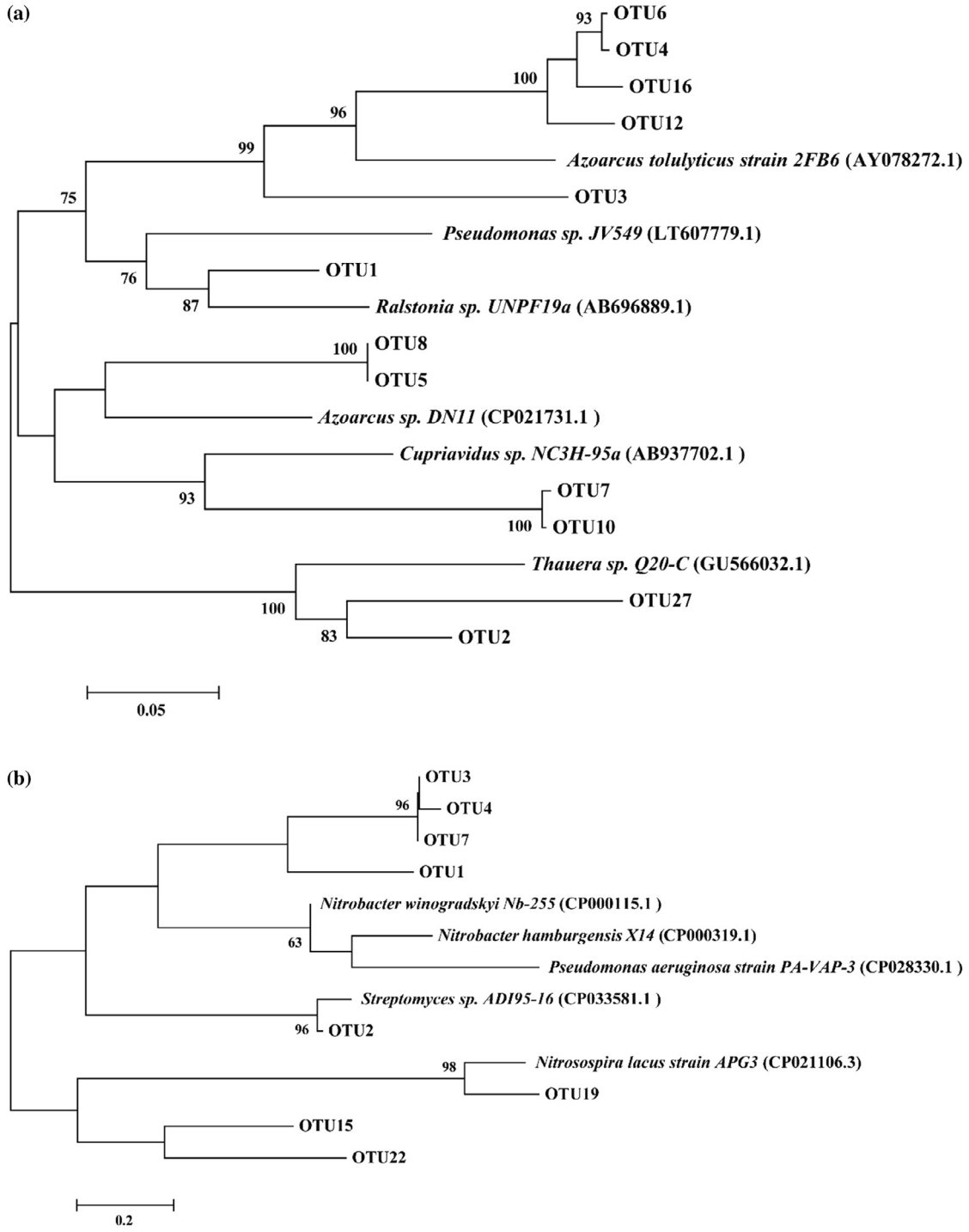

Fig. 3. Phylogenetic trees based on the translated amino acid sequences of the *nirS* (a) and *nxrA* (b) genes from the compost samples and constructed using the neighbor-joining method.

3.4. Roles of communities that harbored nirS and nrxA in the transformation of nitrite

Structural equation modeling (SEM) is an a priori approach that can yield intuitive graphical representations of the complex relationship networks in composting systems (Zhao et al., 2018). Therefore, SEM was employed to explore the role of *nxrA*-like nitrite oxidizers and *nirS*-like nitrite reducer to nitrite conversion. In SEM, a positive correlation demonstrates the mutual promotion of two factors, whereas a negative correlation denotes utilization or formation between them (Wu et al., 2018b). In the present study, it was hypothesized that the structures of the communities that harbored *nxrA* and *nirS* (abundance and composition) could influence the transformation of nitrite.

NO_2^- was found to have a significantly negative influence on the community that harbored *nirS* (Fig. 4a), and the community that harbored *nirS* was significantly related to NO_3^-. The community that harbored *nxrA* had strong negative effects on NO_3^-, and NO_3^- had a significantly positive relationship with NO_2^-. According to the standardized total effects determined by SEM (Fig. 4a), the NO_3^- content was directly affected by the community that harbored *nxrA*, but the community that harbored *nirS* was not directly associated with the oxidization of NO_2^- to NO_3^-, although it was indirectly related to an

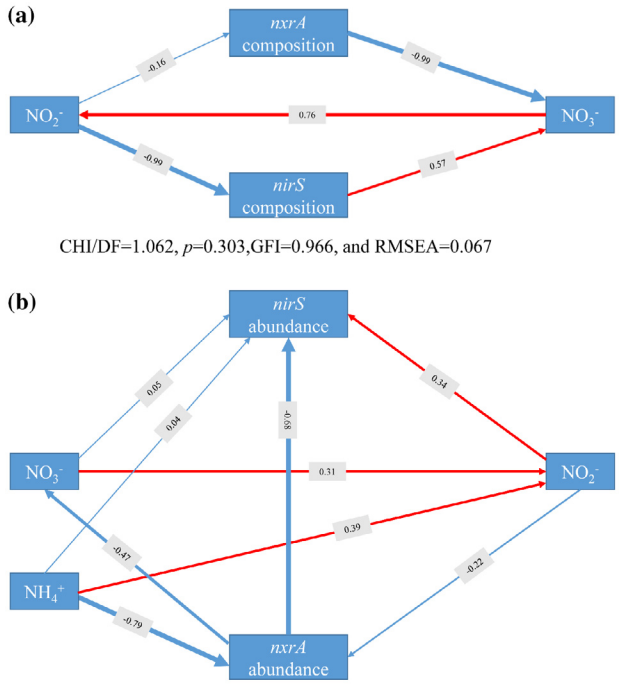

Fig. 4. Structural equation modeling (SEM) showing the relationships between environmental factors and communities that harbored *nirS* and *nxrA* (a), and the abundances of *nirS* and *nxrA* (b). Red and blue arrows indicate positive and negative relationships, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase in the NO_3^- content. A previous study found that the accumulation of NO_2^- retarded the growth of NOB during composting (Fukumoto and Inubushi, 2009). The results obtained in the present study suggest that the community harboring *nirS* could convert NO_2^- into NO, thereby alleviating the toxic effects of NO_2^- on the community that harbored *nxrA*.

Furthermore, path analysis was conducted based on the abundances of the *nxrA* and *nirS* genes with respect to nitrite conversion (Fig. 4b), and the results were consistent with the communities that harbored *nxrA* and *nirS*. The abundance of *nirS* had positive effects on NO_2^- ($\lambda = 0.34$, $P < 0.001$) and NO_3^- ($\lambda = 0.05$, $P < 0.001$), thereby indicating that NO_2^- was related to the increase in the abundance of the *nirS* gene during the composting process, especially when nitrite accumulated in the initial stage of composting because the *nirS* gene is essential for denitrification to reduce NO_2^-. The abundance of *nxrA* had negative effects on NO_2^- ($\lambda = -0.22$), NO_3^- ($\lambda = -0.47$, $P < 0.05$) and NH_4^+ ($\lambda = -0.79$, $P < 0.001$), thereby indicating that some of the NO_2^- was oxidized to produce NO_3^- via the activity of the *nxrA* gene. The NO_3^- content decreased during the first 21 days of composting, even though NOB began to function. In addition, NH_4^+ plays a key role in the NO_2^- conversion process, where it can be converted into NO_2^- by AOA/AOB (Yin et al., 2016), but AOA/AOB grow slowly because the energy yield is low when using NH_4^+ as a substrate (Li et al., 2016a). Therefore, it was assumed that the NO_2^- produced in the early phase of composting was obtained mainly from denitrification. Wang et al. (2018) also showed that nitrate reduction gene products (e.g., *narG*) can mainly reduce NO_3^- to NO_2^- during the mesophilic phase of composting. Thus, the relative contribution of *nirS* to the conversion of nitrite occurred mainly in the early phase of composting, while the *nxrA* and *nirS* genes reached equilibrium in terms of their effects on NO_2^- in the late phase of composting, and *nxrA* had a significantly stronger effect than denitrification (the NO_3^- content increased), especially in the compost maturity phase.

3.5. Relationship between nirS and nrxA contents, gene abundances, and NO_2^- concentration

Comparing ecological networks along environmental gradients can generate new insights into the relative importance of environmental filtering and the coexistence mechanisms that determine community assemblies (Pellissier et al., 2018). The network comprising the *nxrA* and *nirS* genes, NO_2^-, and the bacteria that harbored *nxrA* and *nirS* is shown in Fig. 5. The network had 60 nodes and 377 edges, with a modular structure comprising four major modules. Module 1 was dominated by denitrifiers that harbored *nirS*, where 87.5% of the nodes had positive associations with the *nirS* gene and 75% had positive associations with NO_2^-. The most densely connected node in each module was defined as the "hub" (Li et al., 2015). "OTUs 3, 4, 6, 12, and 16" formed the hub in Module I and these OTUs mainly included *Ralstonia* sp. *UNPF19a* and *Azoarcus tolulyticus strain 2FB6* according to phylogenetic analysis (Fig. 3a). Thus, *Ralstonia* sp. *UNPF19a* and *Azoarcus tolulyticus strain 2FB6* were the main hosts of the *nirS* gene, and the key role of the denitrifiers in module 1 was to reduce the accumulation of NO_2^- during the mesophilic and thermophilic phases. Module 2 was dominated by nitrifiers that harbored *nxrA* where "OTU 2 and 3" formed the hub. Module 2 contained 58.8% nodes that had positive associations with the *nxrA* gene, and these OTUs were mainly represented by *Nitrobacter* and *Pseudomonas*, which oxidized nitrite in the maturity phase. The hubs in Module 3 comprised *nirS*-OTU8 and *nxrA*-OTUs 1 and 5, which mainly acted on nitrite during the cooling phase (Fig. 3b). In Module 3, nitrifiers that harbored *nxrA* and denitrifiers that harbored *nirS* had positive associations, thereby indicating that the relationships between these microorganisms may have been cooperative during the cooling phase (Faust and Raes, 2012). Module 4 comprised nitrifiers that harbored *nxrA* mainly in the maturity phase, but these OTUs included uncultured cluster sequences. Han et al. (2017) also found that some unclassified OTUs could oxidize nitrite in the environment, and some NOB are known to be even more difficult to culture in the laboratory than AOA or AOB.

The OTUs all had positive associations in four separate modules and these positive relationships were probably due to cross-feeding and co-aggregation (Faust and Raes, 2012), thereby suggesting that these microbes acted together to occupy a common niche and they played the same role in nitrogen conversion during the composting process. However, there were also negative relationships between some modules, e.g., 91.9% of the 124 edges between Module 1 and Module 2 had negative interactions, and there were some negative interactions in the 12 edges between Module 1 and Module 3. A previous study showed that negative relationships may be attributed to competition, predation, or amensalism in networks (Faust and Raes, 2012), and the nitrifiers that harbored *nxrA* and the denitrifiers that harbored *nirS* shared a common substrate in the present study (i.e., NO_2^-). Thus, these results may indicate possible competition for resources among the organisms that harbored *nirS* and *nxrA* in the cooling and maturity phases, thereby supporting the SEM results.

4. Conclusion

The results showed that the *nirS* gene was abundant throughout the composting process, whereas the *nxrA* gene was undetectable during the mesophilic and initial thermophilic phases. *Ralstonia* sp. and *Thauera* sp. were the dominant denitrifiers that harbored *nirS*, and *Nitrobacter winogradskyi Nb-255* was the dominant nitrifier that harbored *nxrA*. The NO_2^- that accumulated in the initial composting stage was mainly reduced by dominant denitrifiers that harbored *nirS* in the early phases, and oxidized by nitrifiers that harbored *nxrA* during the late phase, especially in the maturity phase. Bacteria that harbored *nirS* and *nxrA* competed during the cooling and maturity phases.

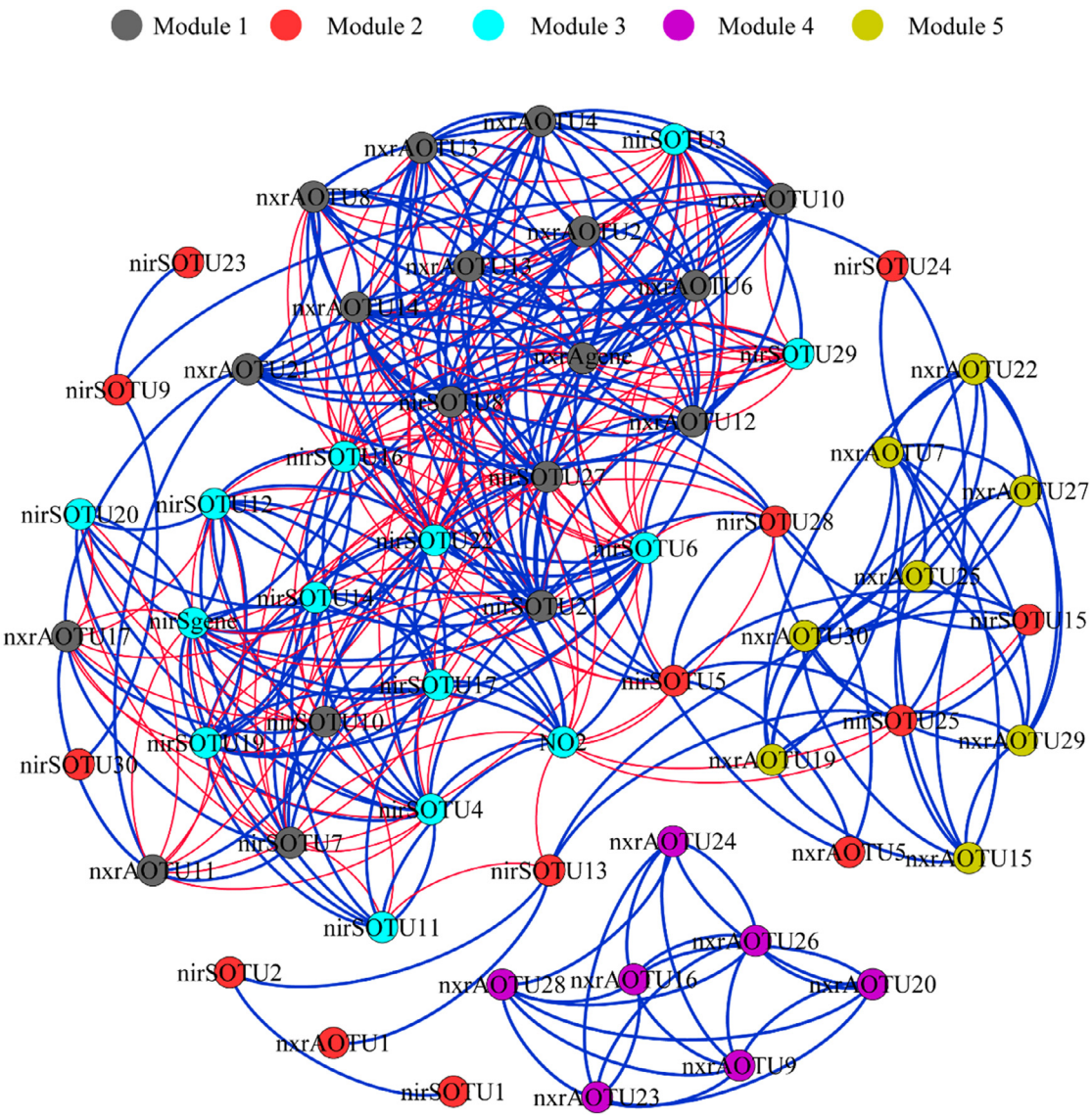

Fig. 5. Network obtained based on the communities that harbored *nirS* and *nxrA*, the abundances of *nirS* and *nxrA*, and NO_2^- showing the co-occurrence and modular patterns during swine manure composting. Blue solid lines represent significantly strong positive ($r > 0.80$) linear relationships and red lines represent strong negative ($r < -0.80$) linear relationships. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Author Contributions

Yanan Yin, Xiaojuan Wang, and Jie Gu designed experiments; Yanan Yin and Chao Yang carried out experiments; Yanan Yin and Wei Zheng analyzed experimental results. Yanan Yin wrote the manuscript. Rong Chen, Xiaochang Wang and Ru Wang improved the quality of manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122426.

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