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ENVIRONMENTAL BIOTECHNOLOGY

Response of denitrifying genes coding for nitrite (*nirK* or *nirS*) and nitrous oxide (*nosZ*) reductases to different physico-chemical parameters during agricultural waste composting

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Abstract The present research was performed to clarify the changes of denitrifying genes (*nirK*, *nirS*, and *nosZ*) abundances under different physico-chemical parameters through evaluating the relationships between the genes abundances and parameters during agricultural waste composting. The genes abundances were determined by real-time quantitative PCR (qPCR). The correlations between physico-chemical parameters and denitrifying genes abundances were analysed by regression analysis. qPCR results showed that the *nosZ* gene abundance was higher than that of *nirK* and *nirS* genes. The *nirK* gene abundance was higher than *nirS* gene indicating that nitrite reducers with Cu-containing enzyme encoded by *nirK* gene were more of importance than those with cytochrome *cd*1 nitrite reductase encoded by *nirS* gene in the nitrite reduction step. Regression analysis suggested that (1) *nirK* gene abundance was correlated with pile temperature following quadratic model; (2) *nirS* gene abundance was linearly correlated with pile temperature and concentration of NH_4^+, while correlated with concentration of NO_3^- and pH following inverse and quadratic model respectively; (3) *nosZ* gene abundance was quadratically correlated with pH and linearly correlated with water soluble carbon (WSC).

Keywords Composting · Denitrifying genes · Physico-chemical parameters · qPCR · Regression analysis

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Introduction

As a well-known method to stabilize organic wastes, composting is obtaining more researches since this biological process can convert biodegradable components into nuisance-free, sanitary and humus-like materials which can be used for organic fertilizer (Kulcu and Yaldiz 2004; Kalemelawa et al. 2012; Wang et al. 2013). Although many solid wastes are disposed economically by composting, 60 % of the total nitrogen (N) can be reduced from compost causing lower fertilizer efficiency and higher levels of harmful gases, such as nitrous oxide (N_2O) and ammonia (NH_3) (Angnes et al. 2013). The vital emission of NH_3 is happened during the thermophilic phase due to strong microbial activity, while the consecutive production of N_2O must be after high-temperature period (Fukumoto et al. 2003; Kuroda et al. 1996; Sommer and Møller 2000).

During the composting process, a part of ammonium (NH_4^+) is transformed into nitrate (NO_3^-) by nitrifiers through nitrification. However, denitrifiers will bring the inverse impact, changing nitrate into nitric oxide (NO), N_2O, and dinitrogen (N_2) progressively making less nitrogen in compost. What is worse, N_2O can be emitted through both

nitrification and denitrification processes as intermediate products and by-products, respectively (Maeda et al. 2010a). N_2O, as a kind of significant trace gas in the atmosphere, has a noteworthy global warming potential by absorbing infrared radiation (Warneck 1999), which is about 298 times that of carbon dioxide according to IPCC (IPCC 2007). N_2O explains approximately 7.9 % of total anthropogenic greenhouse gas emissions with concentration of N_2O increasing at a rate of 0.26 % per year (Forster et al. 2007). Another impact of N_2O is that it contributes to the depletion of stratospheric ozone layer through reacting with oxygen (O_2) to generate nitric oxide (NO) (Crutzen and Ehhalt 1977; Crutzen 1981).

According to the estimate of the relative contributions of denitrification and nitrification to actual N_2O emissions during cow manure composting by Maeda et al. (2010a), denitrification was proved to be the regnant source of N_2O generation, particularly after the turnings. Consequently, studying denitrification and the related microorganisms during composting is of great importance. In detail, denitrification is divided into four reaction steps (Zumft 1997), including the reduction of nitrate to nitrite, the reduction of nitrite to NO, the deoxidation of NO to N_2O, and then ended with the further transformation of N_2O to N_2. All the four reactions are catalyzed by nitrate reductase, nitrite reductase, NO reductase, and N_2O reductase, respectively, encoded separately by *napA* or *narG*, *nirS* or *nirK*, *nor* and *nosZ* (Zumft 1997). Philippot et al. (2007) used functional genes involved in denitrification as genetic markers for studying the denitrifiers abundances, diversities, and functional genes expressions in agricultural soil. According to Guo et al. (2013), the abundances of denitrifying functional genes would be good predictors of denitrification when environmental factors changed in the study of effect of long-term wastewater irrigation on potential denitrification and denitrifying communities in soils. Henderson et al. (2010) pointed out that organic C could affect *nosZ* gene abundance but did not affect *nirS* gene abundance for denitrifiers with *nirS* gene could not compete for the nutrients under the experimental conditions in anoxic soil microcosms amended with glucose and plant residues. Maeda et al. (2010b) found that the *nosZ* diversity and N_2O emission were promoted by using the mature compost during cattle manure composting process, while *nirK* diversity was not promoted, and *nirS* was even absent during composting, suggesting that *nosZ* gene diversity was positively correlated with N_2O emission. While in a study of bacterial denitrifier community abundance in an agricultural field by Dandie et al. (2011), no significant correlations were observed between denitrifier community abundance, structure and N_2O emission, or potential denitrification. Agricultural waste from heavy metal-polluted area contains the amounts of heavy metals (Zeng et al. 2013a, b), which would also influence the denitrifying communities. Although there have been many studies about the denitrifying genes, little information is available about the changes of denitrifiers under different physico-chemical parameters during composting.

In the present research, we conducted an agricultural waste compost pile under experimental conditions in order to clarify the response of denitrifying genes abundances to different physico-chemical parameters. To achieve this objective, three main tasks were completed including (1) the measurement of physico-chemical parameters; (2) detection of the bacterial denitrifying genes abundances; (3) estimation of relationships between denitrifying genes abundances and physico-chemical parameters. *NirK*, *nirS*, and *nosZ*, as the representative functional genes in denitrification pathway, were quantified using quantitative PCR (qPCR) method. We would get a further insight into the bacterial denitrifier communities and their shaping factors during agricultural waste composting.

Materials and methods

Preparation of composting materials

The representative agricultural wastes were collected from suburb of Changsha, China. The chemical characteristics of composting materials are shown in Table 1. Soil was collected and sieved through the 40-mesh screen to get rid of the rough plant debris and added to provide necessary nutrients and enrich the microbial species and populations. The rice straw was air-dried and cut into 10–20 mm lengths, used as the organic materials difficult to be decomposed. Some kinds of vegetables were added as the easy-degradable materials after being air-dried and cut

Table 1 The common characteristics of the composting materials

Materials	Moisture content (%)	TOC (g kg^{-1})	TN (g kg^{-1})	C/N ratio	pH
Soil	26.13	55.1	2.4	22.7	4.73
Rice straw	11.73	428.0	8.8	48.8	–[a]
Vegetables	79.06	97.2	5.0	19.6	–[a]
Bran	14.06	474.1	41.2	11.5	–[a]

[a] Samples not determined. *TOC* total organic carbon; *TN* total nitrogen; C/N, TOC/TN

into 10–20 mm lengths. Air-dried bran was added to adjust C/N ratio of the composting materials.

Composting set-up and sampling

Experimental composting system was set up indoors with a wet weight of about 50 kg in an open box having good heat preservation and lasted for 50 days. The dimension of the box was 0.5 m×0.4 m×0.45 m (length×width×height). The typical composting materials soil, rice straw, vegetables, and bran were blended evenly at a ratio of 8:11:3:2 (fresh weight). The initial organic matter content and C/N ratio were about 60 % dry weight (DW) compost sample and 30:1, respectively (Zhang et al. 2011). To avoid anaerobic environment, the composting pile was turned twice a week during the first two weeks and once a week afterwards. Samples were taken once a day at 9 am on day 1, 2, 3, 4, 9, 16, 21, 26, 33, and 50, respectively. When sampled, three subsamples were taken from different locations of the composting pile. Three subsamples for discerning the dynamic development of bacterial denitrifying genes were mixed and stored immediately at −20 °C before used at each sampling time. Samples for physico-chemical parameters determination were gathered and stored at 4 °C before used. During composting process, moisture content was monitored and adjusted by adding sterile deionized water after sampling to maintain at 55–60 % during first fermentation phase (mesophilic stage and thermophilic stage) and 45–50 % during the second fermentation phase (cooling stage and maturation stage).

Physical and chemical determinations

The pile temperature in the top, middle, and bottom of the composting piles was measured with a thermometer. The pH was determined with a pH meter after mechanically shaking the fresh samples in water suspension at a ratio of 1:5 (*w/v*, sample:water) at 100 rpm for 30 min. After pH determination, the suspension was centrifuged at 12, 000 rpm for 15 min and filtered through a 0.45 μm membrane filter and was used for the water soluble carbon (WSC) measurement using Total Organic Carbon Analyzer (TOC-5000A, Shimadzu, Japan). The samples for ammonium (NH_4^+) and nitrate (NO_3^-) content analyses were shaken with 2 M of KCl solution for 30 min until the inorganic nitrogen compounds were extracted adequately, and then ammonium and nitrate were analyzed by a Continuous Flow Analyzer (FIAstar 5000, FOSS, Denmark). The moisture content was measured by drying 10 g fresh samples at 105 °C for 24 h.

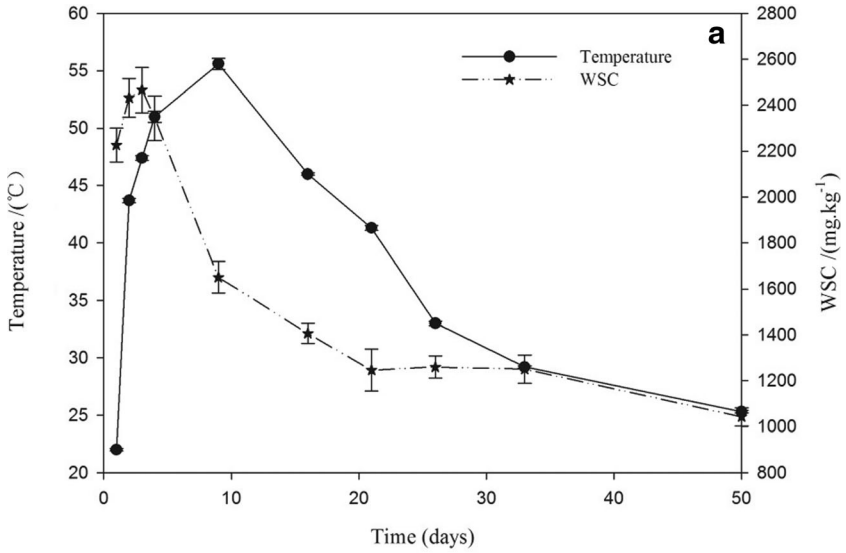
DNA extraction and qPCR analyses

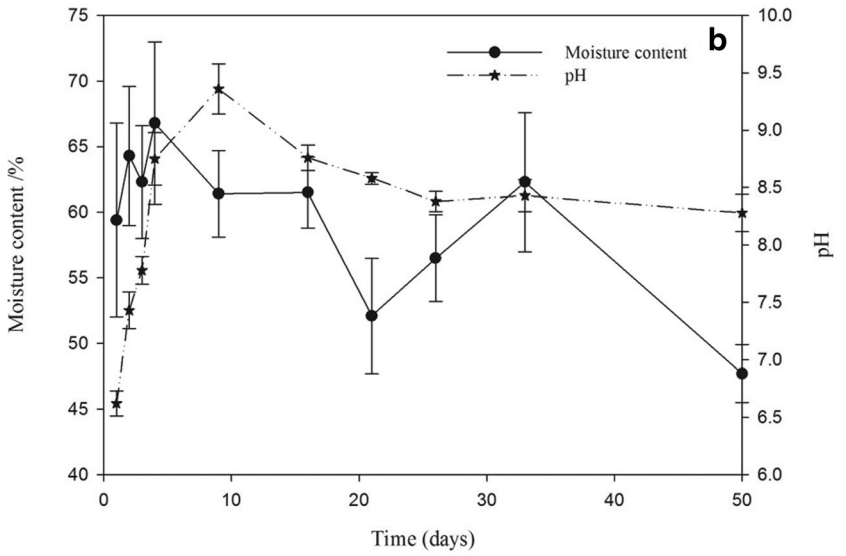
The total genomic DNA was extracted in triplicate from freeze-dried compost samples (~0.5 g) using the PowerSoil kit (MoBio Laboratories, USA) by following the manufacturer's instructions. The DNA extracts were pooled for each sample to reduce sample variability and stored at −20 °C before qPCR. The quantifications of *nirK*, *nirS*, and *nosZ* genes were conducted on an iCycler IQ5 Thermocycler (Bio-Rad, USA) using primers F1aCu and R3Cu (Hallin and Lindgren 1999; Throbäck et al. 2004), cd3aF and R3cd (Zhou et al. 2011), *nosZ*-F, and *nosZ*1622R (Ma et al. 2008), respectively. Each reaction system was performed in a 20 μL volume involving 10 μL of 2×SYBR real-time PCR premixture (Bioteke, Beijing), 0.4 μL (10 μM) of each primer, 2 μL of DNA template, and adjusted with sterile water. Three qPCR reactions were conducted using an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C for *nirK* and *nirS* genes or 40 s at 55 °C for *nosZ* gene, and 40 s at 72 °C. Data were retrieved at 72 °C. These reactions were performed in triplicate for each bacterial denitrifying gene.

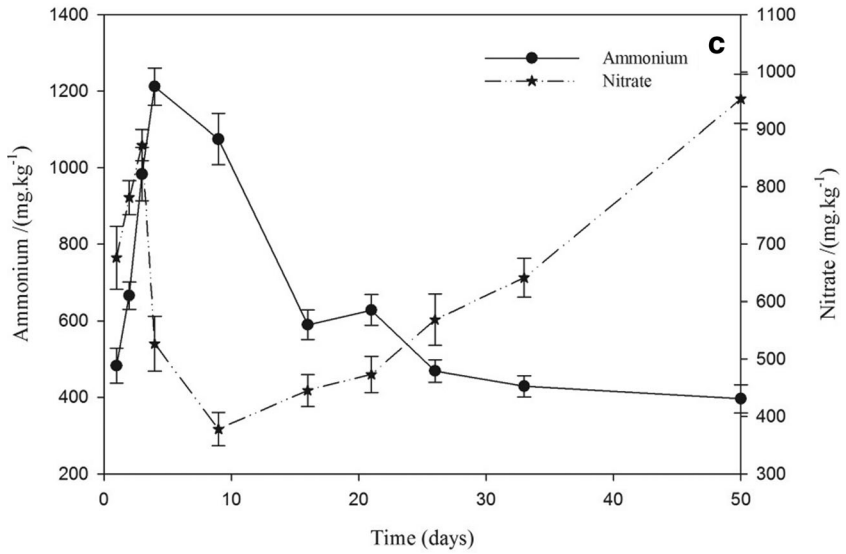
The standard curves for qPCR were produced by ten-fold serial dilutions of linearized plasmids containing cloned *nirK*, *nirS*, and *nosZ* genes obtained from our previous research (Chen et al. 2014). The orders of magnitude of standard curves were 1.0×10^3 to 1.0×10^8 copies of template. All reactions were finished with a melting curve to verify the gene amplification specificity. Inhibitory effects on qPCR performance were detected according to the method described by Zeng et al. (2011). PCR efficiencies and linearity (R^2) of standard curves for *nirK*, *nirS*, and *nosZ* genes were 104.4 % and 2.044, 100.2 % and 2.002, 98.8 % and 1.988, respectively.

Statistical analysis

Three replicate determinations were conducted for the physico-chemical parameters analyses and the mean values were used for further analyses. The maximum difference among triplicate results was below 5 %. Before the further analyses of *nirK*, *nirS*, and *nosZ*, the original data of the three genes abundances were $\log_{10}$-transformed ($\log_{10}(x+1)$). One-way analysis of variance (ANOVA) was performed among parameters using SPSS (version 11.5) to test whether there were any significant differences among means at the 95 % confidence level. Correlation analysis was conducted to obtain the correlation coefficients between parameters and *nirK*, *nirS*, and *nosZ* genes, respectively. According to the results of


a
Temperature
WSC
Temperature /(℃)
WSC /(mg.kg-1)
Time (days)


b
Moisture content
pH
Moisture content /%
pH
Time (days)


c
Ammonium
Nitrate
Ammonium /(mg.kg-1)
Nitrate /(mg.kg-1)
Time (days)

◀ **Fig. 1** Changes of physico-chemical parameters **a** pile temperature and WSC (water soluble carbon); **b** moisture content and pH; **c** ammonium and nitrate during composting process. The *bars* represent the standard deviations of mean values (n=3)

correlation analysis, curve estimation belonging to regression analysis was chosen to evaluate the correlations between each related physico-chemical parameter and the abundances of the bacterial denitrifying genes. Statistical significance was kept at $P<0.05$.

Results

Physico-chemical parameters

The variation of physico-chemical parameters is presented in Fig. 1. As shown in Fig. 1a, the pile temperature increased rapidly once the composting beginning and reached about 47.4 °C on day 3. Thereafter, it reached peak value of 55.6 °C on day 9 during the thermophilic stage (day 4 to day 13, pile temperature exceeding 50 °C). During the cooling stage (day 14 to day 33), temperature dropped from 48 to 29.2 °C before further decreasing to ambient temperature of about 25 °C during the maturation stage (day 34 to day 50). According to Fig. 1a, WSC increased slightly from 2226.8 mg kg^{-1} DW compost sample to 2466.7 mg kg^{-1} DW compost sample on day 3 and dropped to 1042.7 mg kg^{-1} DW compost sample at the end of composting. The moisture content during the first 4 days was adjusted to about 60 %, then the moisture content during composting began to decrease from 67 to 47 % which indicated the degradation of organic matter (Miller and Finstein 1985). The pH increased significantly from 6.62 to 9.36 during the earlier 9 days, and then decreased to 8.28 gradually in the end of composting (Fig. 1b). Figure 1c states the variation of NH_4^+ and NO_3^- content. NH_4^+ content rose rapidly during the first 4 days and soared to the peak value of 1211.5 mg kg^{-1} DW compost sample on day 4 because of the increase of temperature and pH, as well as ammonification and mineralization of organic nitrogen. Afterwards, the value began to decrease gradually until the end of composting with a level of 395.8 mg kg^{-1} DW compost sample. Nevertheless, NO_3^- content increased a little in the first 2 days and then decreased sharply to 377.6 mg kg^{-1} DW compost sample on day 9. Thereafter, the values increased gradually to 953.0 mg kg^{-1} DW compost sample at the end of composting.

Quantification of denitrifying genes

The abundances of *nirK*, *nirS*, and *nosZ* genes are presented in Fig. 2. Obviously, all the three genes were detected during the whole composting ranging from 1.2×10^6 to 3.3×10^7, 3.7×10^5 to 7.1×10^6, and 1.6×10^6 to 3.2×10^7 gene copies g^{-1} DW compost sample, respectively. For *nirK* gene abundance, it increased from 1.2×10^6 to 2.0×10^7 gene copies g^{-1} DW compost sample in the first 3 days and decreased until the 9th day. Afterwards, it increased again to 3.3×10^7 gene copies g^{-1} DW compost sample on day 16 followed by deceasing to 2.9×10^6 gene copies g^{-1} DW compost sample in the end of composting. For *nirS* gene, the abundance increased from 3.7×10^5 to 7.1×10^6 gene copies g^{-1} DW compost sample within the first 9 days, then it decreased to 2.7×10^5 gene copies g^{-1} DW compost sample in the end. The abundance of *nosZ* gene increased to 3.2×10^7 gene copies g^{-1} DW compost sample on day 21 and decreased to 2.9×10^7 gene copies g^{-1} DW compost sample in the end of composting. On the whole, *nosZ* gene abundance was higher than *nirK* and *nirS* genes.

Relationships between abundances of denitrifying genes and physico-chemical parameters

The correlations between parameters and abundances of each denitrifying gene were different (Table 2) according to the results of correlation analysis. *NirK* gene abundance was only correlated with pile temperature (r=0.623, $p<0.05$). While the *nirS* gene abundance was related with pile temperature (r=0.832, p=0.001), NH_4^+ (r=0.733, $p<0.01$), NO_3^- (r=−0.721, $p<0.01$), and pH (r=0.705, $p<0.05$) strongly, respectively. As for the *nosZ* gene abundance, it was found to be related with pH (r=0.768, p=0.005) and WSC (r=−0.691, p=0.013), respectively.

To choose the best suitable models of regression analysis, curve estimation was performed between genes abundances and sample properties. The results are showed in Figs. 3, 4, and 5 for *nirK*, *nirS*, and *nosZ* genes, respectively. *NirK* gene abundance was correlated with pile temperature following the quadratic model with the R^2 of 0.648 and P of 0.026. However, there was a significant positive linear relationship between *nirS* gene abundance and pile temperature with R^2 of 0.692 and P of 0.003 (Fig. 4a). The same relationship was found between *nirS* gene abundance and NH_4^+ with different R^2 of 0.537 and P of 0.016 (Fig. 4b). Inverse relationship was existed between *nirS* gene abundance and NO_3^- with R^2 of 0.621 and P of 0.007 (Fig. 4c). As Fig. 4d showed, *nirS* gene abundance was quadratic correlated with pH of samples

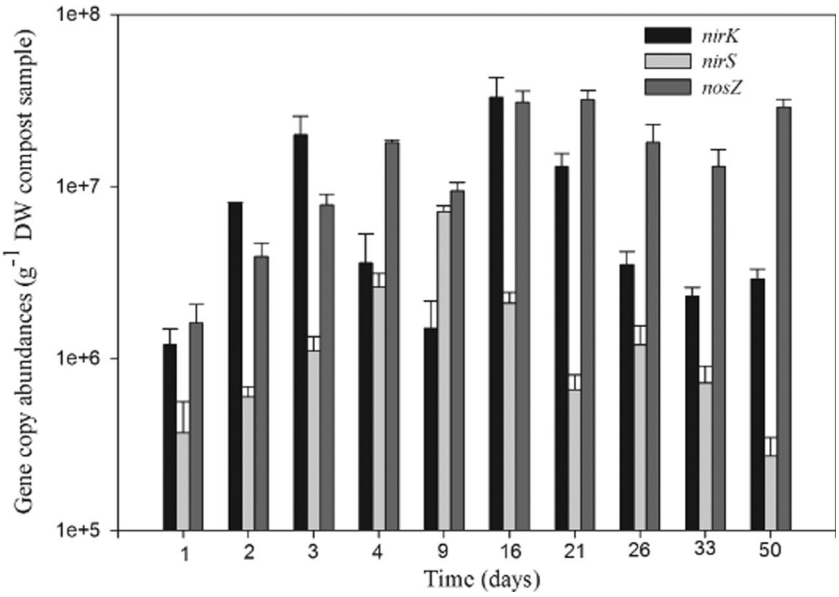


Fig. 2 Denitrifying genes (*nirK*, *nirS*, and *nosZ*) copy abundances during agricultural waste composting process. The *bars* represent the standard deviations of mean values (n=3)

(R^2=0.665, P=0.022). For *nosZ* gene abundance, it was correlated with pH and WSC following the models of quadratic and linear, respectively (Fig. 5). The R^2 between *nosZ* gene abundance and pH was 0.811 and P was 0.003, while R^2 was 0.478 and P was 0.027 between *nosZ* gene abundance and WSC.

Discussion

In the present study, qPCR of genes encoding the key enzymes involved in denitrification (*nirK* and *nirS* encoding copper and cd_1 nitrite reductases and *nosZ* encoding the nitrous oxide reductase, respectively) was carried out to estimate the abundances of functional bacterial communities during agricultural waste composting. The relationships between physico-chemical parameters and denitrifiers were analyzed to determine shaping factors affecting the changes of bacterial denitrifying communities. In this research, the higher abundances of the three genes were detected during the cooling and the mature stages while lower abundances during the mesophilic and the thermophilic stages. This might be due to the increase of NO_2^- concentrations during the composting which provided enough nitrite available for denitrifiers (Wang et al. 2013) and suggested that the shift of temperature affected the denitrifier community (Gödde and Conrad 1999; Holtan-Hartwig et al. 2002). The average copy numbers were in order of *nosZ*, *nirK*, and *nirS*. By comparing the average level of the *nirK* and *nirS* genes copies, it was suggested that nitrite reducers with Cu-containing enzyme encoded by *nirK* gene might be more of importance than those with cytochrome *cd*1 nitrite reductase encoded by *nirS* gene in the nitrite reduction step during the composting process. This was similar with some previous studies (Chen et al. 2010; Maeda et al. 2010b;

Table 2 The correlations between denitrifying genes and physico-chemical parameters

Key genes	Pile temperature		Ammonium (mg kg^{-1})		Nitrate (mg kg^{-1})		pH		WSC	
	r	p	r	p	r	p	r	p	r	p
nirK	0.623	0.025	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
nirS	0.832	0.001	0.733	0.008	−0.721	0.009	0.705	0.011	–[a]	–[a]
nosZ	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	0.768	0.005	−0.691	0.013

[a] Correlations between genes abundances and parameters are not significant

WSC water soluble carbon

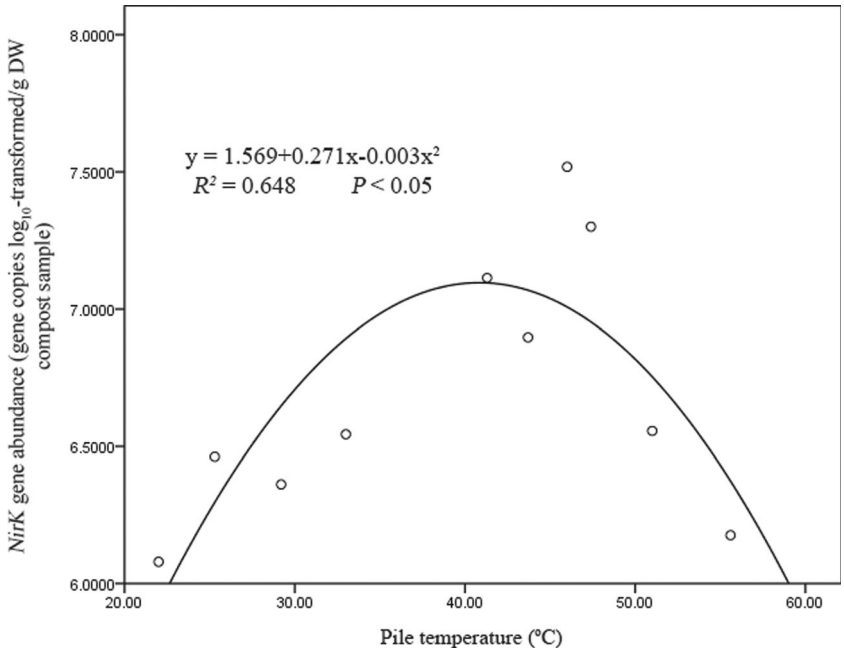


Fig. 3 Relationship between *nirK* gene abundance and pile temperature

Yoshida et al. 2009; Zhou et al. 2011). Yoshida et al. (2009) observed that the abundance of *nirK* was always greater than that of *nirS* in the rice paddy field soil. Bárta et al. (2010) also found that the number of denitrifiers containing *nirS* was two orders lower than that of *nirK* denitrifiers. As for the *nosZ* gene copies, there were several reports indicating that the abundance of *nosZ* was higher than that of *nirK* and *nirS*. In the research conducted by Kandeler et al. (2006), the relative abundances of *nirK*, *nirS*, and *nosZ* were 0.2, 0.4, and 0.5 %, respectively, indicating the average abundance of *nosZ* was persistently the highest during the composting process. According to Zumft (1997), denitrifiers contain either a cytochrome *cd*1 nitrite reductase or a Cu nitrite reductase and the abundance of *nirK* or *nirS* is lower than that of *nosZ*. However, some researchers found some interesting phenomena different from ours (Dandie et al. 2011). Chon et al. (2011) observed the gene copy numbers of the *nirS* gene were higher than those of *nosZ* gene in both February and May at all the sampling sites. In the studies of Dandie et al. (2008) and Henry et al. (2006), the gene copy numbers of *nirK* showed higher than that of *nosZ*. No matter what the order of *nirK*, *nirS*, or *nosZ* gene copy numbers is, it may not indicate that the one showing higher abundance contributes more to denitrification during composting than the other denitrifiers (Philippot and Hallin 2005). The studies of mRNAs level will allow further insights into denitrification activity and monitoring of the active denitrifiers than single indication of their presence (Philippot and Hallin 2005).

Correlation coefficients were determined to access the correlations between physico-chemical parameters and the abundance of *nirK*, *nirS*, or *nosZ*. The results showed that the abundance of *nirK* shared a positive correlation with pile temperature. The *nirS* gene abundance shared strong correlations with pile temperature, NH_4^+ and NO_3^-, and pH. While for *nosZ*, there were strong correlations between *nosZ* gene abundance and pH and WSC. The different effects on the three genes were results of adaption to environmental conditions. The importance of pile temperature changes for dynamics of the denitrifier community has been stated by several studies (Gödde and Conrad 1999; Holtan-Hartwig et al. 2002). In the present study, *nirK* and *nirS* genes abundances were quadratically and linearly correlated with temperature, respectively, suggesting that the *nirK* gene abundance was more sensitive to pile temperature. When the temperature was above 40 °C, the growth of *nirK* gene might be suppressed. WSC was widely recognized as the necessary nutrients for all the heterotrophic bacteria to undertake metabolism in the composting. Kandeler et al. (2006) indicated that the amount of organic substance was positively related to the three denitrification genes in soil of Glacier Foreland. Zhou et al. (2011) also supplied evidence that organic matter could stimulate soil denitrifiers as the important substrate. However, WSC was only negatively correlated with *nosZ* gene abundance during the composting in this study, which might be due to the degradation of organic compounds by heterotrophic bacteria, while the population of denitrifier increased with time.

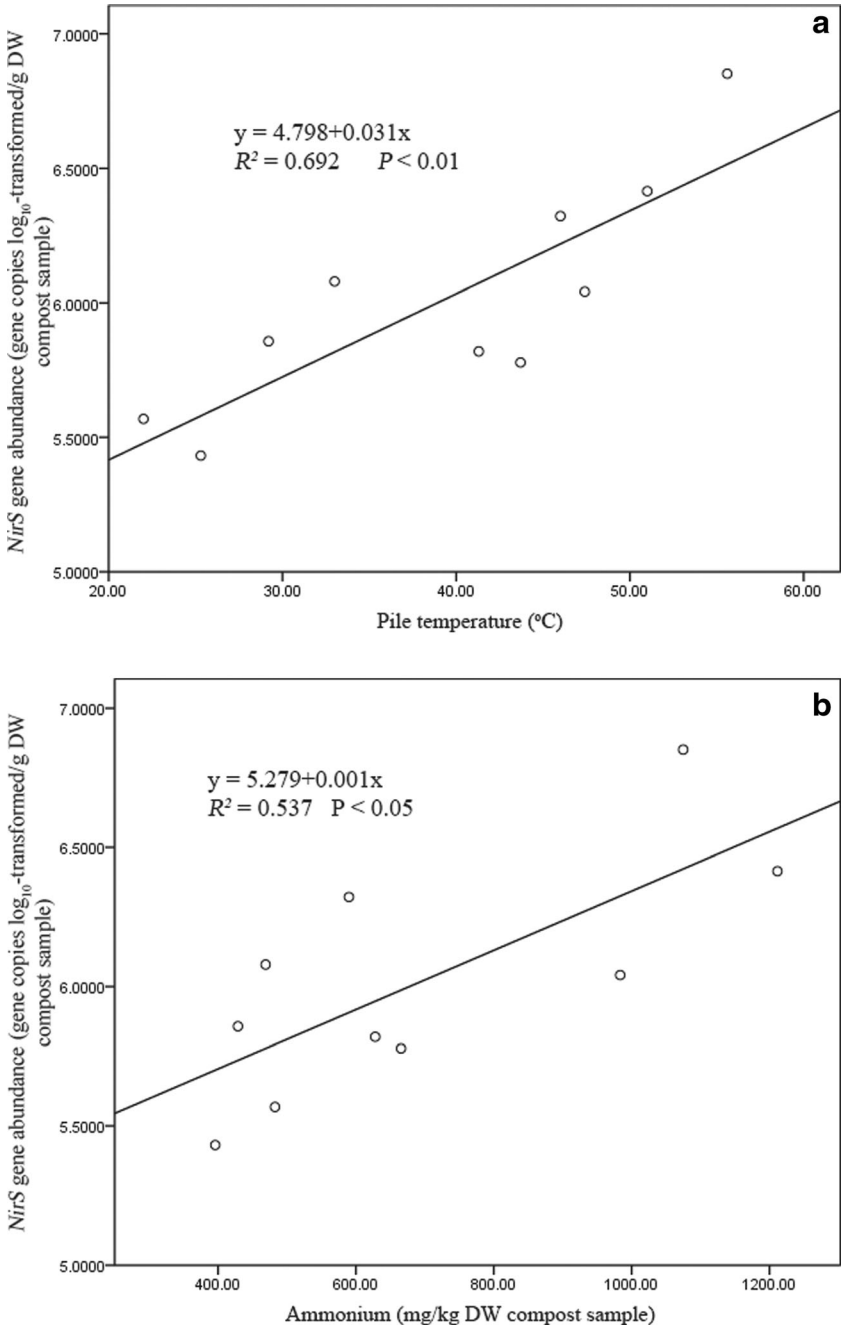


Fig. 4 Relationships between *nirS* gene abundance and **a** pile temperature and **b** ammonium and **c** nitrate and **d** pH, respectively

In this study, pH increased rapidly from 6.62 to 9.36 which might be due to the release of NH_3 and mineralization of organic nitrogen, and then decreased to 8.28 in the end. As an important parameter, previous studies found that pH could significantly affect the denitrifying communities (Deiglmayr et al. 2004; Tiquia 2002). These results were in agreement with ours approximately. In this present study, both of *nirS* and *nosZ* genes abundances were quadratically correlated to pH. The difference was that *nirS* gene abundance decreased to the minimum value firstly and then increased following the rise of pH. While the relationship between *nosZ* gene abundance and pH was contrary. This phenomenon might be attributed to the different microbial metabolism under different pH. However, the results indicated that the alkaline condition (pH>7.5) in the compost pile might be more comfortable and adaptable for *nirS* and

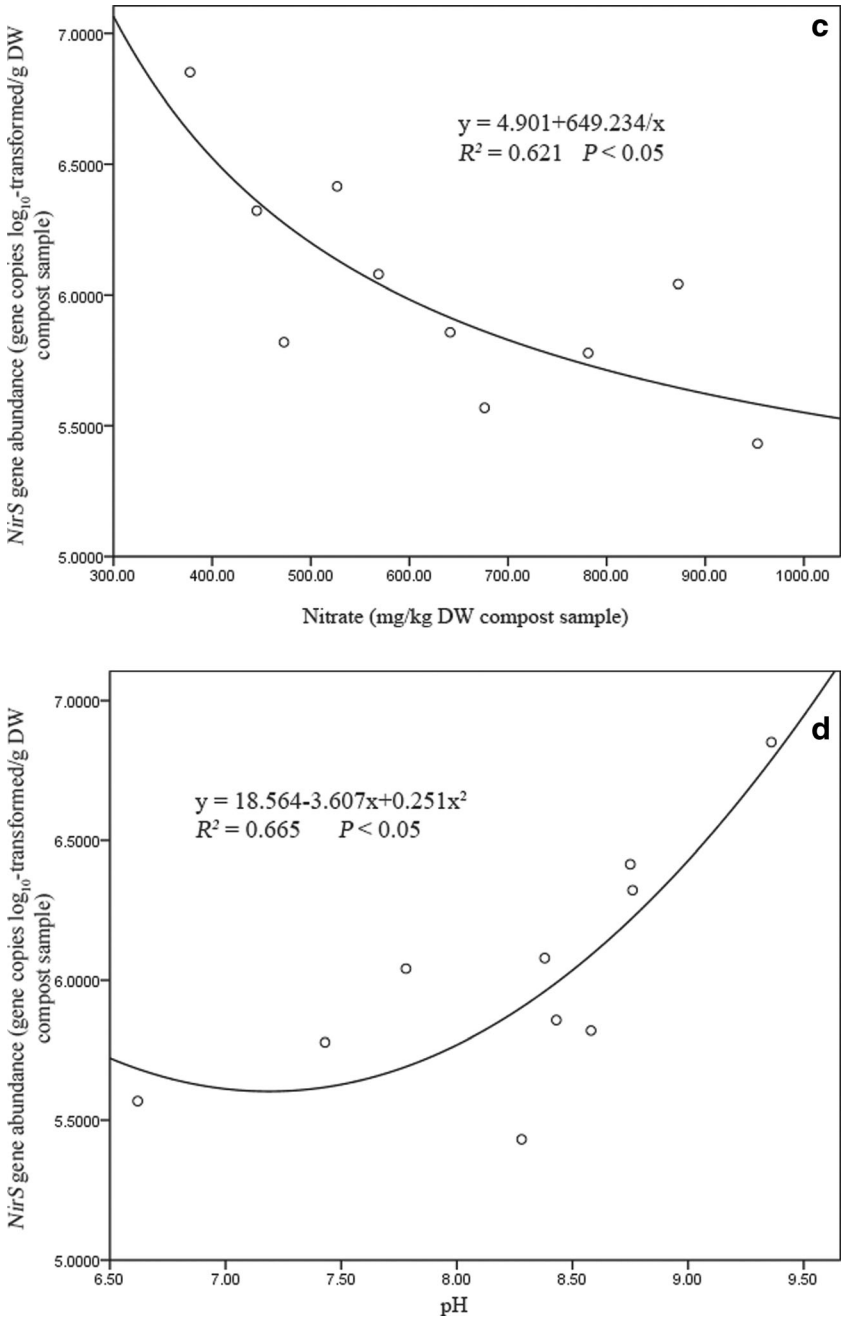


Fig. 4 (continued)

nosZ genes. Bárta et al. (2010) found higher *nirK* gene copies in the more alkaline soils and higher *nirS* gene copies in the more acidic soils. However, some other studies reported that there were no pH effects on denitrifying communities. Kandeler et al. (2006) reported no relationships between *nirS*, *nosZ*, and pH.

As the electron acceptor in the step of denitrification, nitrate has been acknowledged to be an important influence factor of denitrifier communities and denitrification rates (Dambreville et al. 2006; Kemp and Dodds 2002). However, the opposite results have also been found in some previous studies suggesting that there were little correlations between nitrate content and denitrifier abundance (Kandeler et al. 2006; Mergel et al. 2001). In our study, the nitrate was inversely related to the *nirS* gene abundance significantly, while correlations

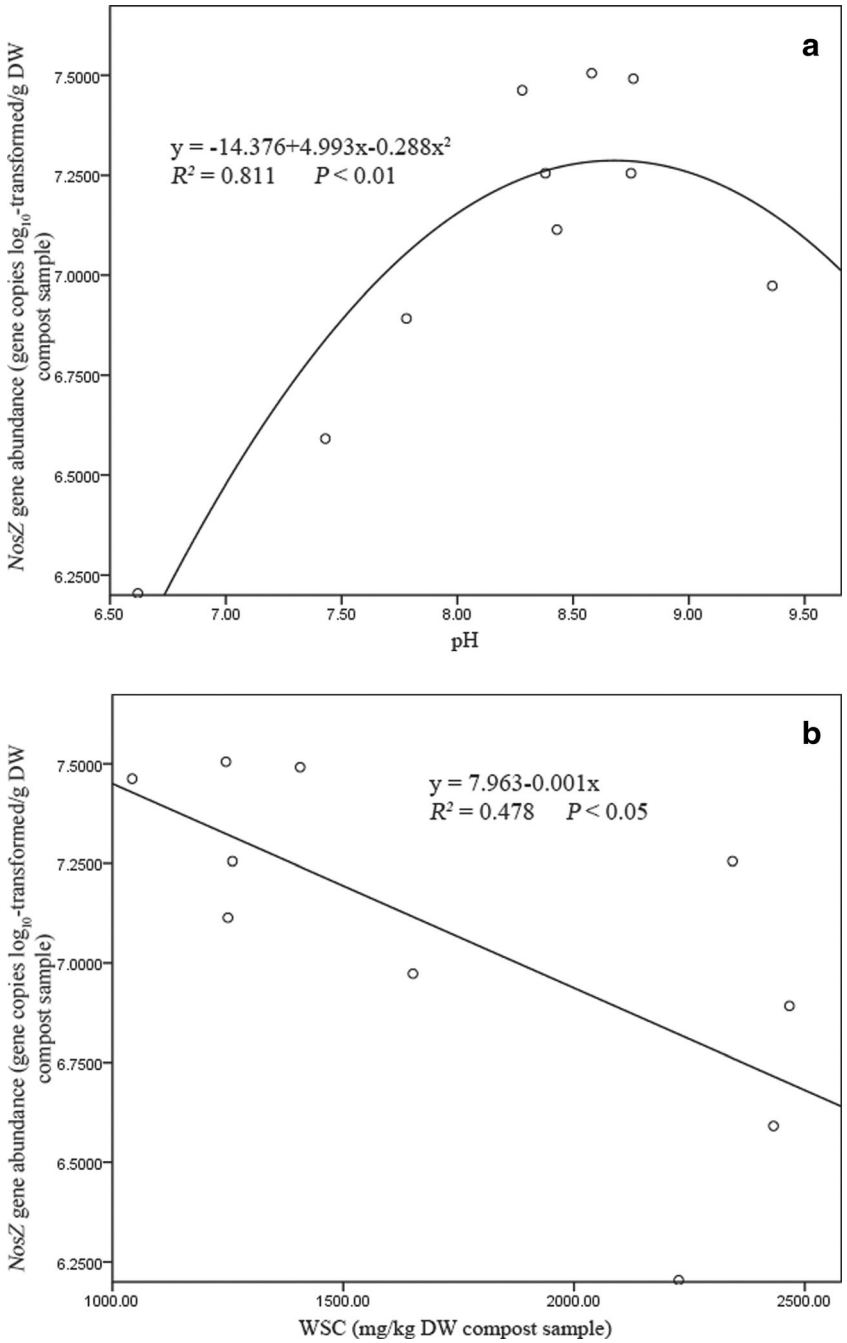


Fig. 5 Relationships between *nosZ* gene abundance and **a** pH and **b** WSC (water soluble carbon), respectively

were found neither between *nirK* gene abundance and nitrate nor between *nosZ* gene abundance and nitrate. This result might suggest that excessive nitrate content would suppress the growth of denitrifiers containing the *nirS* gene during this agricultural composting progress, which would support Liu et al. (2003), who once found that the denitrification rate and unique clones of *nirS* were the lowest in the sediments with highest NO_3^- concentrations. Avrahami et al. (2002) found the populations of denitrifiers were affected by the addition of ammonium to soils. Similarly, ammonium was linearly correlated with *nirS* gene abundance in this study. A further study of mRNA levels of denitrifying genes is necessary to get a better insight into the changes of bacterial denitrifying genes under different environmental parameters.

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