Abundance of AOA, AOB, nirS, nirK, and nosZ in red soil of China under different land use

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Abstract. In this study, four land use type soils from Yingtan Jiangxi Province China, i.e., forest (F), bamboo (B), tea plantation (TP) and upland (U), were collected, and gene of ammonia-oxidizing archaea (AOA) and bacteria (AOB), nirS, nirK, and nosZ were determined, to identify the effects of land use on abundance of microorganism in red soil and their role to nitrification and denitrification. The result shows that AOA copy numbers ranged from 6.20 x 10^6 to 6.58 x 10^6 copies/g soil and AOB varied from 4.18 x 10^6 to 7.41 x 10^6 copies/g soil. The highest AOA and AOB were all measured in U soil that was the highest pH and the lowest C/N ratio. The Abundance of AOB is stimulated by enhancing soil pH due to lime application and more available NH4+ from N fertilization that could be responsible for the high net nitrification rate in U soil. Meanwhile, nirK copy numbers ranged from 6.46 x 10^6 to 7.05 x 10^6 copies/g soil, nirS from 5.50 x 10^6 to 5.85 x 10^6 copies/g soil, and nosZ from 6.57 x 10^6 to 7.35 x 10^6 copies/g soil. The nirS (p<0.05) and nirK (p<0.05) was positively correlated with soil potential denitrification rate.

1. Introduction
Nitrogen, as one of the crucial nutrients for all organisms [1] and a macro component required by plants, plays an essential role in biogeochemical cycles and shows a fundamental part in controlling of ecosystem structure and function [2,3]. Sustainability of ecosystem services would be unlikely to anticipate as long as have no understanding of how environmental factor influence N transformations [4]. Better appreciating of N transformation in soils could expose the ability of biological inorganic N supply, expand the efficacy of N fertilizers [5,6] and determine N dynamic and cycling [7] that is needed at regional and smaller scales [8].

Nitrification and denitrification are two crucial N transformation processes in the soil-plant system [9]. Nitrification that produces nitrate (NO3-), gaseous nitrous oxide (N2O) or proton, is a two-step aerobic process in which ammonium (NH4+) is oxidized to nitrite (NO2-) and nitrite is further oxidized to nitrate [10]. Ammonium monooxygenase (AMO) encoding key enzymes the first step of
nitrification (i.e., the reduction of $\text{NH}_3^+$ to $\text{NH}_2\text{OH}$) which is composed of three subunits encoded by genes of $\text{amoA}$, $\text{amoB}$, and $\text{amoC}$, respectively [11]. Since Winogradsky confirmed the role of bacteria in mediating the initial step of the nitrification pathway in 1890, AOB were considered organisms capable of nitrification. Therefore from 1890 until 2004, scientists believed that only bacteria mediated aerobic ammonium oxidation. In 2005, the concept changed by the discovery of ammonia-oxidizing archaea (AOA) [12] also as potential players [13]. However, the relative significance of AOB and AOA in ammonia oxidation is still not totally identified, and their relative contribution to nitrification may vary, depending on soil conditions [14].

Meanwhile, denitrification as the process by which nitrogen in the form of nitrate ($\text{NO}_3^-$) and nitrite ($\text{NO}_2^-$) is transformed to nitric oxide (NO) that influence to Ozon structure, nitrous oxide ($\text{N}_2\text{O}$) as green-house gas and di-nitrogen ($\text{N}_2$), plays a significant role in the N cycle [15,16]. Denitrifying microorganisms use $\text{NO}_3^-$, $\text{NO}_2^-$, NO or $\text{N}_2\text{O}$ as electron acceptors and reduce them to $\text{NO}_2^-$, NO, $\text{N}_2\text{O}$ or $\text{N}_2$. Knowles [17] stated that the reduction of nitrite to nitric oxide, for denitrification process, is catalyzed by two forms of nitrite reductases (Nir). $\text{nirS}$ is one gene that encodes a cytochrome-containing enzyme ($\text{cdl}$–Nir). The other gene is $\text{nirK}$ that encodes a copper-containing enzyme (Cu-Nir). If the denitrification proceeds to completion, $\text{N}_2\text{O}$ is reduced by nitrous oxide reductase, which can be encoded by the gene $\text{nosZ}$.

Different land-use types could have differences on managing agricultural soils. Management practices, such as fertilization and amendment as a soil reparation way, promote the activity of nitrifying bacteria (i.e. AOA and AOB as two fundamentally different groups of microorganisms [18]) in the agricultural soils, thus increasing the production of $\text{NO}_3^-$ [19–22]. The application of N fertilizer and lime as several kinds of management practices and land-use could significantly stimulate the relative contribution of bacteria and fungi to specific soil nitrification activities[23].

Land-use types can significantly affect soil organic carbon content and composition, soil pH, microbial properties and other soil properties, which may affect denitrification [24]. Xu and Cai [16] suggested almost in contrast that not pH as a key factors, land-use and management practices favor soil carbon and nitrogen accumulation and anaerobic activities enhance soil denitrification capacity. Besides, Knowles [17] determined main factors controlling denitrification such as organic carbon, oxygen, nitrogen oxides, pH, temperature, and inhibitors. Soil organic matter, as the main energy source and electron donor of soil microorganisms, is an essential factor affecting denitrification. It is generally believed that the higher the soil organic matter, the greater the denitrification potential [25]. Moreover, the form of organic carbon also affects the denitrification potential of soil. Studies have shown that the mineralized carbon is more closely related to the denitrification potential than the total organic carbon content, and the soil denitrification is affected by the anaerobic mineralization Carbon [26].

Area of the subtropical region of China is of a quite large size that covers amount to 166,900 km$^2$ (account for 14.8% of the country’s subtropical area) [27]. Xu and cai [16] stated acidic soils cover large of the humid subtropical region of China (pH <5.5). Lou et al. [28] argued one of the typical agricultural soils in subtropical China is called red soil that is classified as Ultisols and some of the Alfisols and Oxisols in the soil taxonomy of USA and according to Wilson et al. [29] occasionally as Alfisols, Mollisols and even Inceptisols.

The red soil region is an important grain and economical crop production area in China and plays a vital role in agricultural production. Over the past 60 years, characteristics of the subtropical region of China is significantly agricultural. Because of rapid alterations of land-use patterns to meet the increasing demand for food, cash crops, and fiber [27]. Numerous studies have been conducted on the soil transformation process in China’s subtropical region [23,30,31]. For example, Wang et al. [23] have shown previous studies that land-use and management practices in the humid subtropical region in China could impact the relative contribution of bacteria and fungi to specific soil nitrification processes. It has also been verified that physical and chemical properties of the humid subtropical region soil are obviously influenced by the use of inorganic nitrogen fertilizer, organic manure, and lime [32], the organic carbon content is generally small due to intensive land-use and poor
management. Liming that widely practiced in this region, raises soil pH that is essential for crop production in acidic soils. Further soil acidification is caused by planting tea due to the physiological features of tea plants [33]. However, the expanded knowledge and further work, especially about nitrifiers and denitrifiers associated with land-use effect, are needed.

In this study, the abundance of AOA, AOB, nirS, nirK, and nosZ under different land-use types in the red soil of China were determined and the presence of their encoding genes was measured by real-time quantitative polymerase chain reaction (qPCR).

2. Material and method

2.1. Site Description and soil samples
The study site was located in Yingtan City, Jiangxi province, a red soil typical region in China. Its typical climate is categorized as the subtropical monsoon. The precipitation is 1785 mm (30-year average) annually. The mean annual temperature of the site is 18.4°C, with a maximum average monthly temperature of 29.9°C in July (30-year average).

The natural vegetation consists of a mixed forest of oak (Quercus Mongolia), Chinese red pine (Pinus massoniana Lambo), and China fir (Cunninghamia lanceolata (Lamb.) Hook.), and at present, the main land-use types are forest land, tea garden, bamboo forest, upland, and rice fields. Chemical fertilizers are intensively applied to rice fields, upland, and tea gardens, and the upland has the tradition of applying lime to improve acid soil. Urea is also often applied to rice paddies, while a large amount of ammonium bicarbonate is applied to the uplands [34]. Four kinds of land use types of forest land (F), tea garden (TP), bamboo (B), and upland (U) were selected, and each of the four different sites was selected as three repetitions, totaling 12 sampling sites.

Four kind of land use soils were selected in October 2017 to investigate the effect of land use on nitrifier and denitrifies. Three soil samples from different land-use (a depth of 0-20 cm) took from each site. After sampling, the soils were immediately sieved (<2 mm), kept moist and stored at 4°C in sealed plastic bags before use for experiment and before further analysis. All samples were, divided into 3 subsamples, one subsample stored at 4°C for the incubation studies, one subsamples was dried to determine the physical and chemical properties of the soil (Table 1), and one subsample was stored in a -80°C refrigerator, for extracting DNA to determine soil microbial properties.

2.2. Analysis of Soil Properties and gene abundance
Soil pH was determined by DMP-2 mV/pH meter (Quark Ltd, Nanjing, China), soil: water ratio was 1:5 (v/v); soil organic carbon determined by potassium dichromate volumetric method; soil total nitrogen The measurement was carried out using a semi-micro-Kelvin method.

Extraction of soil DNA and detection of ammonia bacteria (AOB) and ammonia-oxidizing archaea (AOA) amoA gene abundance: 0.5 g of dry weight soil in a -80 °C refrigerator, using PowerSoil® DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), the extraction of total soil DNA was performed according to the instructions. AOB and AOA amoA gene abundance were determined by quantitative polymerase chain reaction (qPCR) method, using SYBR green dye method, the detection equipment was Bio-Rad CFX96 (Bio-Rad Laboratories, USA), and each soil sample was assessed three times. DNA was extracted in parallel. The qPCR reaction system contained 10 μl of 2×SYBR (Premix Ex Taq (TaKaRa, Dalian, China), 0.5 μM upstream and downstream primers and 1 μl of DNA template (9.0-23.7 ng). Makeup to 20 μl with MilliQ water. Melting curve analysis was used to determine the specificity of the amplified product. The procedure was from 65 to 95 °C, and the fluorescence value was read after 0.5 °C increase per cycle. A cloning plasmid of the corresponding gene in the environmental DNA sample was selected as a standard DNA template. The DNA template concentration of qPCR standard curve of AOB and AOA was 1.60×102 -1.60×108 and 1.27×102 -1.27×108 copies/μl, respectively, and the amplification efficiency was 96.4-101.5% (R2 =
0.995-1.000). A series of DNA template dilutions were used to assess whether PCR was inhibited during amplification.

Primers for quantifying the denitrification function genes nirK, nirS, nosZ are shown in Table 1. The amplification conditions for quantitative PCR were pre-denaturation at 95 °C for 2 minutes, denaturation at 95 °C for 10 seconds, annealing at 58 °C for 20 seconds, extension at 72 °C for 20 seconds, and 40 cycles.

| Targeting gene | Primer set | Sequence (5'-3')³ |
|----------------|------------|-------------------|
| nirK           | nirK1F     | GGMATGGTKCCSTGGCA |
|                | nirK5R     | GCCCTGATCAGRTTRTG |
| nirS           | Cd3aF      | GTSAACGTSAAGGARCSGG |
|                | R3cd       | GASTTCGGRTGSGTCTTG |
| nosZ           | nosZBb     | AACGCCTAYACSACSTGTT |
|                | nosZRb     | TCCATGTGCAGNGCRTGGCAGA |

Note: a bold letter indicates the location of the degenerate base.

2.3. Statistical Analysis
One way analysis of Variance (ANOVA) and Duncan’s test were used to determine significant differences in soil gene abundance and other determined soil properties. All statistical analyses were performed using SPSS software package 23.0 for Windows.

3. Result
3.1. Soil properties
All soils are categorized as acidic soil, and the soil pH (H₂O) ranged from 4.49 to 4.84 (Table 2). The lowest soil pH was measured in bamboo soil (B), and the highest one was measured in the upland soil (U). The soil organic C (SOC) and total N in U was the lowest compared to the forest, bamboo, and tea plantation. The SOC concentrations ranged from 9.46 to 30.47 g C kg⁻¹.

| Soil Properties | Land Use Types |
|-----------------|----------------|
|                 | F   | TP | U  | B   |
| pH              | 4.60 ± 0.15ab | 4.51 ± 0.08b | 4.84 ± 0.11a | 4.49 ± 0.05b |
| SOC (g kg⁻¹)    | 21.23 ± 4.43b | 30.47 ± 2.05a | 9.46 ± 0.20c | 25.10 ± 1.66b |
| TN (g kg⁻¹)     | 0.98 ± 0.11c  | 2.25 ± 0.14a  | 0.83 ± 0.04c | 1.62 ± 0.13b  |
| C/N             | 21.51 ± 3.06a | 13.54 ± 0.29bc| 11.48 ± 0.57c| 15.49 ± 0.35b |
| CO₂ (mg C/kg)   | 297.4 ± 37.3bc| 389.2 ± 58.5b | 235.1 ± 50.7c| 561.51 ± 39.2a |

Data are shown as mean ± S.D.; SOC = Soil organic carbon; TN = Total nitrogen; the same letter in the same line indicate there is no significant difference at p<0.05 level.
The highest SOC and total N concentrations were measured in tea plantation soil (TP). The TN concentrations ranged from 0.83 to 2.25 g N kg⁻¹. The highest TN concentration was also measured in TP. The soil C/N ratio was between 11.48 and 21.51. The lowest soil C/N ratio was measured in U, and the highest one was measured in B. Generally, both SOC and TN concentrations were in the following order: TP ˃ B ˃ F ˃ U and soil C/N ratio was in the following order: F ˃ B ˃ TP ˃ U.

During anaerobic culture (method is not shown), the cumulative CO₂ emissions from F, TP, U, and B were 297.4, 389.2, 235.1 and 561.1 mg C/kg, respectively. The soil of TP was significantly higher than that of the other three types of soil, and the cumulative CO₂ emissions from U soil is lowest.

3.2. Ammonia oxidizer abundance (AOA and AOB) in the soil

The archaeal amoA gene (AOA) copy numbers of four different land-use were between $6.20 \times 10^6$ and $6.58 \times 10^6$ copies/g soil. The AOA abundance in U was significantly higher than B, but there was no significant difference among U, TP, and F. The copy numbers of bacterial amoA gene (AOB) ranged from $4.18 \times 10^6$ copies/g soil to $7.41 \times 10^6$ copies/g soil (Figure 1). The AOB abundance was significantly different among the different land-use (p < 0.05). The highest AOB was measured in U, and the lowest was in B. The AOB abundance in different land-use was in the following order: U ˃ TP ˃ F ˃ B (Figure 1). The AOB abundance increased with the increase in pH (p<0.05), while decreased with the increase in the C/N ratio (p<0.05).

![Figure 1](image.png)

**Figure 1.** AOA and AOB amoA genes copies numbers of four different land-use types. Error bars represent the standard deviation of the mean

3.3. nirS, nirK and nosZ Gene abundance

We examined nirS and nirK gene as nitrite reductase and nosZ as nitrous oxide reductase (Figure. 2). The nirS gene was $5.50 \times 10^6$, $5.86 \times 10^6$, $5.57 \times 10^6$ and $5.85 \times 10^6$ for F, TP, U, and B, respectively. The higher nirS numbers were observed at TP as much $5.85 \times 10^6$ copies g⁻¹ soil and the lowest one is F soil, but no significant differences for among of soils.

The nirK gene was $6.51 \times 10^6$, $6.79 \times 10^6$, $6.46 \times 10^6$ and $7.05 \times 10^6$ for F, TP, U, and B, respectively. The nirK gene was more abundant than nirS. The higher nirK numbers were observed at
B as much 7.05 x 10^6 copies g^-1 soil, and the lowest one is U soil. Among the four different land use showed significantly different, especially between B and TP.

Meanwhile, the nosZ gene was 7.13 x 10^6, 7.16 x 10^6, 6.57 x 10^6 and 7.35 x 10^6 for F, TP, U, and B, respectively. The nosZ gene generally was more abundant than both nirS and nirK. The higher nosZ numbers were observed at B as much as 7.35 x 10^6 copies g^-1 soil, and the lowest one is U soil. Among four different land use showed significantly different for U soil only.

Figure 2. The abundance of denitrifying genes (nirS, nirK, and nosZ) in different land-use types in Jiangxi Province, China. Different letter on bars indicates significant differences (Duncan test p<0.05). Error bars are standard deviations.

4. Discussion
The different land-use types could have differences in soil organic C, total N, soil temperature, and other soil properties [30,35]. Zhang et al. [30] stated that land-use change leads to transform management practices including application of inorganic N fertilizer, organic manure, lime and cultivation that in turn affect to both chemical and physical soil properties. Acidic soil of the subtropical region of China was used to maximize yield during its intensive cultivation. Acid soil infertility is a significant factor that limits crop production in red soil, as China’s essential land resources, regions of southern China [29]. Liming is introduced as management practices to overcome acidic soil on supporting and optimizing crop yield. The primary effect of liming of soil is to increase pH as also was reported [23] that at the beginning of agricultural land establishment, lime addition often enhanced soil pH. Besides, the rapid mass application of N fertilizer in the subtropical region of China improved N soil availability.

Meanwhile, intensive cultivation reduced and moved quickly out litterfall. It is moving out of litterfall that is controlled by plantation species reduced biological activity on the decomposing process and creating C availability in soil. As a result, N availability much higher as compared to C availability in the agricultural soil. In this study, upland soil that represents agricultural land use indicated the highest in soil pH and the lowest in C to N ratio.

The differences in significant soil properties such as pH, soil N status, and other soil characteristics tend to affect the population abundance and growth of AOB and AOA. Generally, trend of the abundance of AOA and AOB amoA gene in agriculture soils was AOA predominate among AOB and
symbolize the most plentiful ammonia-oxidizing organisms in soil ecosystems on Earth [18]. In this study, copy numbers of AOA amoA gene were higher than those of AOB amoA gene for forest, tea plantation and bamboo forest soil (in line with Leininger et al. 2006), with the exception for upland soil. Both of AOA and AOB amoA gene in upland soil were more exceptional than other land-use types and, AOB amoA gene outnumbered to AOA amoA gene. Our results showed that the AOB abundance increased with the increasing in pH (p<0.05), while decreased with the increasing in the C/N ratio (p<0.05). Thus lime addition that enhanced soil pH was likely a crucial factor affecting AOB abundance in the upland soils. Previous investigations also found that AOB was sensitive to soil pH and positively correlated with soil pH [19,20,36]. N fertilization management was another critical factors regulating AOA and AOB abundance in agricultural soils [36,37], as also already reported by Alam et al. [38] that long-term fertilization led to increased abundance of amoA gene in AOA and AOB. These results were in agreement with finding that AOB prefers a high N environment [39]. Our results showed that AOB abundance in upland soils was significantly higher than the land use soils. Previous studies also reported that N fertilizer could stimulate more than 10-fold of the growth of AOB in the soil [19,21].

Our results showed that AOB was significantly correlated with soil pH, in line with the previous investigation that also found the increasing AOB with increasing pH [40]. Despite net nitrification rate (data is not shown) were positively correlated with both AOB (p<0.01) and AOA (p<0.05), the stronger correlation between net nitrification rate and AOB indicated that AOB was likely more essential factors regulating nitrification rate among different land use than AOA. Jiang, Hou [41], using soil from the same region, also confirmed that AOB but not AOA were significantly correlated with soil pH, and also potential nitrification activity was more strongly correlated with AOB than AOA.

The abundance of nirS gene was positively correlated with the cumulative CO₂ emission (p=0.05); the abundance of nirK gene was positively correlated with total soil organic carbon (p<0.05) and CO₂ cumulative emissions (p<0.01); nosZ gene abundance also showed a significant positive correlation with total soil organic carbon (p<0.05) and CO₂ cumulative emissions (p<0.05) (data is not shown). Denitrification potential (data is not shown) and soil organic carbon (p<0.05), cumulative CO₂ emission during anaerobic culture (p<0.01), nirS gene abundance (p<0.05) and nirK gene abundance (p<0.05) were a significantly positive correlation. Studies have reported a significant positive correlation between the abundance of nirK, nirS and, nosZ functional genes and pH [42]. However, the relationship between nirK, nirS and nosZ functional gene abundance and pH was not found in the results of this study; and stepwise regression analysis found that soil CO₂ cumulative emissions and organic carbon content are key factors controlling the abundance of nirK and nosZ genes, respectively. Therefore, the abundances of denitrifying functional genes nirS, nirK, and nosZ were significantly positively correlated with CO₂ cumulative emissions, further supporting conclusions that the mineralized C was the critical factor controlling the potential denitrification among the studied land use soils (i.e. soil-organic carbon, primarily the easily-mineralized carbon characterized by CO₂ cumulative emissions, is the main reason for the difference in denitrification potential of red soil in different land-use types).

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