

## Effect of Soil pH on Composition and Abundance of Nitrite-oxidising Bacteria

Mansur Abdulrasheed<sup>1\*</sup>, Hussein I. Ibrahim<sup>2</sup>, Fatima Umar Maigari<sup>3</sup>, Ahmed F. Umar<sup>4</sup> and Salihu Ibrahim<sup>5</sup>

<sup>1</sup>Department of Microbiology, Gombe State University, P.M.B. 127 Gombe, Nigeria.

<sup>2</sup> School of Biological Sciences, Queen's University Belfast 2017, University Road, Belfast, BT7 1NN, Northern Ireland, United Kingdom.

<sup>3</sup>Department of Biochemistry, Gombe State University, P.M.B. 127 Gombe, Nigeria.

<sup>4</sup>Abubakar Tafawa Balewa University, Bauchi State, Nigeria.

<sup>5</sup>Centre for Biotechnology Research, Bayero University, PMB 3011, Kano, Nigeria.

\*Corresponding author:

Mansur Abdulrasheed,

Department of Microbiology Gombe State University,  
Gombe Nigeria,

PMB 127 Gombe Nigeria.

Email: [moladeji@yahoo.com](mailto:moladeji@yahoo.com)

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### ABSTRACT

Nitrification, the microbial oxidation of ammonia to nitrate (NO<sub>3</sub><sup>-</sup>) via nitrite (NO<sub>2</sub><sup>-</sup>) is a vital process in the biogeochemical nitrogen cycle and is performed by two distinct functional groups; ammonia oxidisers [comprised of ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA)] and nitrite oxidising bacteria. Autotrophic nitrification is said to occur in acidic soils, even though most laboratory cultures of isolated ammonia and nitrite oxidising bacteria fail to grow below neutral pH. Published studies revealed that soil pH is a major driver for determining the distribution and abundance of AOB and AOA. To determine whether distinct populations of nitrite oxidising bacteria within the lineages of *Nitrospira* and *Nitrobacter* are adapted to a particular range of pH as observed in ammonia oxidising organisms, the community structure of nitrite oxidising bacteria within the lineages of *Nitrospira* and *Nitrobacter* were determined across a pH gradient (4.5 – 7.5) by amplifying nitrite oxidoreductase (*nxrA*) and 16S rRNA genes followed by denaturing gradient gel electrophoresis (DGGE). The community structure of both *Nitrospira* and *Nitrobacter* changed with soil pH, with distinct populations observed in acidic and neutral soils. The abundance of *Nitrospira*-like 16S rRNA and *Nitrobacter*-like *nxrA* gene copies contrasted across the pH gradient. *Nitrobacter*-like *nxrA* gene abundance decreased with increasing soil pH, whereas *Nitrospira*-like 16S rRNA gene abundance increased with increasing pH. Nitrification activity of both *Nitrospira*-like and *Nitrobacter*-like NOB in acidic and neutral pH soil was investigated in a microcosm experiment incubated at 30 °C for 21 days, with high rates of nitrification observed in both soils. Findings indicated that abundance and distributions of soil NOB is influence by soil pH.

### INTRODUCTION

Soil microorganisms are the most abundant and diverse group of organisms on earth, and their composition and diversity in nature is thought to have a direct influence on wide range of ecosystem processes. However, soil pH is known to have great influence on the diversity and composition of soil bacteria communities. Soil pH is considered as a factor regulating the concentration, bioavailability and the chemical form of nutrients in soil [1]. It was attested that community structure and bacterial diversity on global scale is strongly determined by soil pH [2], and it is also

believed that both the activity and distribution of archaeal and bacterial ammonia oxidisers are influenced by soil pH [3]. Investigation of soil microcosms revealed the mechanisms by which soil pH affect the growth and activity of microbial functional groups.

For instance, rates of nitrification. Thus, in acidic soil the rate of ammonia oxidation can occur at low rates [4] and in liquid batch culture below pH 7 ammonia oxidising bacteria show little or no growth [5]. The reduction in the activities and growth of ammonia oxidisers at lower pH is attributed to lower availability

of ammonia, as pH decreases due to an increased in ammonium via ionization [6]. Autotrophic nitrification occurs in acid soils, even though laboratory cultures of isolated ammonia and nitrite oxidising bacteria fail to grow below neutral pH. It was thought that ammonia oxidation may be possible in acid soils through biofilm and aggregate formation [7] and increased urease activity [8], also allows growth below pH 5.

However, recent evidence show that acidophilic thaumarchaeal ammonia oxidiser (*Nitrosotalea devanattera*) grow maximum at pH range 4 to 5.5 [9]. Soil pH is considered as a major driver of both bacterial [10] and archaeal [3] ammonia oxidiser community structure. In addition, *amoA* genes of both archaea and bacteria have also been retrieved from soils with pH ranging from 3.7 [11] to 8.65 [12]. Also, soil pH tends to lead to selection for distinct archaeal and bacterial communities [9,3,13,10], thereby inactive adaptation and selection of specific phylogenetic groups in acidic soils [4].

Most of the previous research on the diversity and abundance of nitrifiers have focused on ammonia oxidisers: ammonia oxidising bacteria [AOB] and ammonia oxidising archaea [AOA], as the first step of autotrophic nitrification is assume to be rate limiting [3]. However, nitrite has been shown to accumulate in soil with high pH and ammonia concentrations via inhibition of nitrite oxidoreductase by ammonia. In addition, steamed sterilized soil [14] and drought [15] inhibit nitrite oxidation more than ammonia oxidation.

There is significant published work on ammonia oxidising bacteria and ammonia oxidising archaea that demonstrates soil pH as the key driver of adaptation, evolution and diversity of ammonia oxidisers. However, no work has been performed investigating the effect of soil pH on nitrite oxidising bacteria that perform the second crucial phase in nitrification. Thus, importantly the functional roles exhibited by the two major nitrite oxidising bacteria encountered in soil, *Nitrospira* and *Nitrobacter*, requires a better understanding.

This study is therefore, aimed at examining the role of soil pH on the composition and abundance of nitrite oxidising bacteria. In order to examine whether soil pH influence these groups of organisms, a comprehensive understanding of the ecology of nitrite oxidising bacteria is required. This study will therefore, investigate whether distinct populations of nitrite oxidising bacteria within the lineages of *Nitrospira* and *Nitrobacter* are adapted to a particular range of pH as observed in ammonia oxidising organisms by investigating the communities over an established pH gradient.

## MATERIALS AND METHODS

### Soil Sample Collection

Soil samples were collected in triplicate across a pH gradient of 4.5-7.5 from an experimental agricultural plot (Scottish Agricultural College, Craibstone, Aberdeen, Scotland, Grid reference NJ 872104) that contain a series of sub-plots each of which have been maintained for 52 years with a gradient of pH values of approximately 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 [16]. Three samples of 200 g were collected at random from each sub-plot, sieved (3.35 mm mesh size) and then stored at -20 °C and 4 °C, respectively, before DNA extraction, physicochemical analysis and microcosm construction.

### Soil Chemical Analysis

For each individual replicate, total carbon, nitrogen, pH, and water content was determined. Soil pH was measured using a glass electrode. A thermo Quest NCS analyser (NCS 2500) was used to determine both total carbon and nitrogen content, while organic matter was determined as loss of ignition at 430 °C for 24 h using a dry combustion muffle furnace (GallenKamp, England). Water content was determined as the weight loss after drying at 100 °C for 24h.

### Soil Microcosms and Analysis

Twelve replicate microcosms consisting of 8 g of fresh soil were constructed and sealed with a rubber stopper in 125 mL sterile serum bottles for two sets of soil samples at pH 4.7 and 7.5 plots (24 in total) for destructive sampling in triplicate. Microcosms were incubated at 30 °C and destructive sampling was carried out on day 0 and after 7, 14, and 21 days of incubation. During incubation, aerobic conditions were maintained by briefly removing the seal and venting for 10 minutes every two days.

Soil collected from destructive sampling was immediately frozen and stored at -20 °C before chemical analysis and nucleic acid extraction. Combined nitrite+nitrate concentration was determined colorimetrically by flow injection analysis (FIA star 5010 Analyser, Hoganas, Sweden) after extraction from 5 g of wet soil in 30 mL of 1 M KCl.

### Nucleic Acid Extraction and Quantification of Nitrite Oxidising Bacteria

Nucleic acids were extracted from 0.5 g of soil as previously described [17]. The purity of the extracted DNA solutions (final volume, 30 µL) was checked using a NanoDrop spectrophotometer (ND-1000) and were diluted to 2 ng/µL and 10-fold with sterile distilled water and used as template for qPCR and PCR respectively. *Nitrobacter*-like and *Nitrospira*-like NOB abundance was determined by quantitative PCR targeting the functional gene *nxrA* coding for nitrite oxidoreductase and 16S rRNA genes in *Nitrobacter* and *Nitrospira* respectively, as described by [18].

*Nitrobacter*-like *nxrA* genes were amplified using primers F1*nxrA* and R2*nxrA* [19] and the 16S rRNA genes of *Nitrospira* were amplified using primers Nspra675f and Nspra746r [20], recently used for soil samples [21] as shown in **Table 1**. Amplification was performed using a Real Plex<sup>2</sup> Master cyclor (Hamburg, Germany).

Each reaction was performed in a 20 µL volume, containing 1 µL each of both primers, 10 µL of QuantiTech SYBR Green PCR Master Mix (Qiagen) and 5 µL of nucleic acid, while *Nitrospira* reaction volume contains 1 µL each of both primers and 10µL of QuantiFast<sup>TM</sup>qPCR Master Mix (Qiagen). Thermal cycling conditions are described in (**Table 1**). Standard curves were obtained using two replicates of serial dilutions [10<sup>1</sup>-10<sup>8</sup>] containing cloned *Nitrobacter*-like and *Nitrospira*-like sequences respectively.

The standard curve descriptors [slopes, efficiencies (E) and determination coefficient (R<sup>2</sup>)] were as follows: *Nitrobacter*-like *nxrA* gene copy numbers: slopes -3.0, E= 1.15 and R<sup>2</sup>=0.989, and *Nitrospira*-like 16S rRNA gene copy numbers: slope -3.3, E = 0.99 and R<sup>2</sup>= 0.999. At the end of each qPCR run, a melting curve analysis was performed to check for both amplification and specificity of qPCR products. And the amplicons were run on agarose gel to also further check for specification.

**Table 1.** Summary of primers and PCR conditions

Primers	Sequence 5' – 3'	Ref	PCR Thermal cycling conditions
F1 <i>nrxA</i>	CAGACCGACGT GTGCGAAAG	[19]	15min at 95°C followed by 45 cycles at 95°C for 1 min, 30 s at 58°C annealing temperature and elongation at 72°C for 1 min, 80°C for 8s, 95°C for 15s, 60°C for 15s and 95°C for 15s.
R2 <i>nrxA</i>	TCCACAAGGAA CGGAAGGTC	[19]	15min at 95°C followed by 45 cycles at 95°C for 1 min, 30 s at 58°C annealing temperature and elongation at 72°C for 1 min, 80°C for 8s, 95°C for 15s, 60°C for 15s and 95°C for 15s.
Nspra675f	GCGGTGAAAT GCGTAGAKATC G	[20]	5min at 94°C followed by 40 cycles at 94°C for 30 s, 1 min at 64°C annealing and elongation at 81°C for 8s, then 95°C for 15s, 60°C for 1s, and 95°C for 15s.
Nspra746r	TCAGCGTCAGR WAYGTTCCAG AG	[20]	5min at 94°C followed by 40 cycles at 94°C for 30 s, 1 min at 64°C annealing and elongation at 81°C for 8s, then 95°C for 15s, 60°C for 1s, and 95°C for 15s.
m27f	AGAGTTTGATC CTGGCTCAG	[25]	5min at 95°C followed by 10 cycles of at 94°C for 30 s, annealing at 58°C and elongation at 72°C for 45s, followed by 25 cycles of 92°C for 30 s, 58°C for 30 s with terminal elongation at 72°C for 45s.
Nspra746r	TCAGCGTCAGR WAYGTTCCAG AG	[25]	5min at 95°C followed by 10 cycles of at 94°C for 30 s, annealing at 58°C and elongation at 72°C for 45s, followed by 25 cycles of 92°C for 30 s, 58°C for 30 s with terminal elongation at 72°C for 45s.
F1 <i>nrxA</i> -GC	CAGACCGACGT GTGCGAAAGC <u>GCCCGCCGCG</u> <u>CGCGCGGGG</u> <u>GGGGCGGGG</u>	[19]	5min for at 95°C followed by 35 cycles at 94°C for 30 s, annealing at 61°C and elongation at 72°C for 1 min with terminal elongation at 72°C for 5min
CTO189f	GGAGAAAAGC AGGGATCG	[25]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C
CTO654r	CTAGCYTTGTA GTTTCAAACGC	[25]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C
CrenamoA2 3f	ATGGTCTGGCT WAGACG	[26]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C
CrenamoA6 16r	GCCATCCATCT GTATGTCCA	[26]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C
357f-GC	<u>GCGCCGCGCG</u> <u>GCGCGCGGG</u> <u>CGGGCGGGG</u> <u>GCACGGGGG</u>	[23]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C
518r	ATTACCGGGC TGCTGG	[23]	5min at 95°C followed by 10 s at 94°C for 30 s, 55°C at 30 s, 72°C at 1 min, followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min then 10 min at 72°C

Note: Sequence underline corresponds to GC-clamp

### DGGE Analyses

PCR-DGGE analysis was used to characterize the community structures of *Nitrobacter*-like and *Nitrospira*-like NOB by targeting *nrxA* and 16S rRNA genes respectively. However, it has been reported that to date, there is no molecular tool available for targeting specifically *Nitrospira*-like NOB, using a functional gene.

The communities of AOB and AOA were also characterized. *Nitrobacter*-like *nrxA* sequences were performed using two PCR steps and primers as previously described by [19].

Briefly, primary amplification was performed with F1*nrxA* and R2*nrxA* same primers as for qPCR, the purified PCR products were then used as a template for the secondary amplification with the same primers containing a GC clamp added at the 5' end of the forward primer [19].

*Nitrospira*-like 16S rRNA genes were amplified using m27f and Nspra746r [22] specific to the genus *Nitrospira*, a dominant group of soil NOB [21]. The second PCR was performed using the amplification product of the first PCR as template with 357f-GC [containing a GC clamp] and 518r primers [23]. All the amplification products were diluted 1:20 prior to secondary amplification to prevent primers carryover and the amplification of non-target sequences. All the PCR runs were performed in 0.6 ml tubes, the reaction mixtures containing; 1 x reaction buffer, 1 µL of DNA extract, 1 µL of each primer, 0.5 µL of 25 mmolNTPs, 2 µL of MgCl<sub>2</sub>, 1 µL of BSA and 0.2 µL of AmpliTag Gold® DNA polymerase (Applied Biosystem).

Ammonia oxidising bacteria communities were characterized by DGGE analysis of 16S rRNA genes as previously described by [24]. 16S rRNA genes of bacterial ammonia oxidiser were amplified using nested-PCR approach [24], with CTO189f and CTO654r primers [25] for the first PCR step and in the second step of PCR amplification the amplicons were nested with P3 (357f-GC) and P2 (518r) [23]. Amplification of archaea *amoA* genes were performed using *CrenamoA23f* and *CrenamoA616r* primers, without attaching a GC clamp to the primers [26]. Amplicons were run on agarose gel to check for amplification of products (Table 1).

DGGE was performed using Dcode Universal Mutation Detection System [Bio-Rad, Hertfordshire, UK] as described previously [27]. PCR products were loaded onto 8% polyacrylamide gels containing a linear gradient of 35-70% denaturant for 16S rRNA gene of *Nitrospira*, *Nitrobacter nrxA* gene and bacterial ammonia oxidiser 16S rRNA gene assays and 15-55% for archaea *amoA* gene assays. Gels were run for 900 min at 100V in 6.5L of 1 x TAE buffer at 60°C constant temperature and silver stained as described previously [27] and then scanned using an Epson GT9600 scanner with transparency unity [Epson, Hemel Hempstead, UK].

### Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine whether soil physicochemical parameters varied between each pH plots. Paired t tests were used to determine whether nitrifier abundances differed across pH gradient and Pearson's test was used to determined wheather nitrite + nitrate concentration was significantly correlated with gene abundances of *Nitrospira* and *Nitrobacter*.

## RESULTS

### Physicochemical Analysis

The measured pH values of each subplot were; 4.7, 5.1, 5.7, 6.2, 6.7, 7.1 and 7.5 (SE < 0.1) with 0.2 maximum deviation from the target pH in any plot. All the measured soil physicochemical parameters with an exception of organic matter showed no variability between plots and no significant trends were observed with pH, as determined by analysis of variance.

### *Nitrobacter*-Like and *Nitrospira*-Like Gene Abundance

Quantification of *Nitrobacter*-like *nrxA* and *Nitrospira*-like 16S rRNA genes by qPCR showed different pH effects on their abundances across each plot. *Nitrobacter nrxA* gene abundance (g<sup>-1</sup> dry soil) decreased significantly with increasing soil pH across the gradient ( $p = < 0.001$ ) whereas *Nitrospira* 16S rRNA

gene copies ( $\text{g}^{-1}$  dry soil) increased with increasing pH (Fig. 1a). The *Nitrospira* gene copies increased to a maximum at pH 6.2 before decreasing slightly at pH 6.7, 7.1 and 7.5 respectively ( $p < 0.001$ ) (Fig. 1b), *Nitrobacter*-like *nxrA* genes were more abundant in acidic pH and decreased significantly as the pH tend to neutral 7.5.

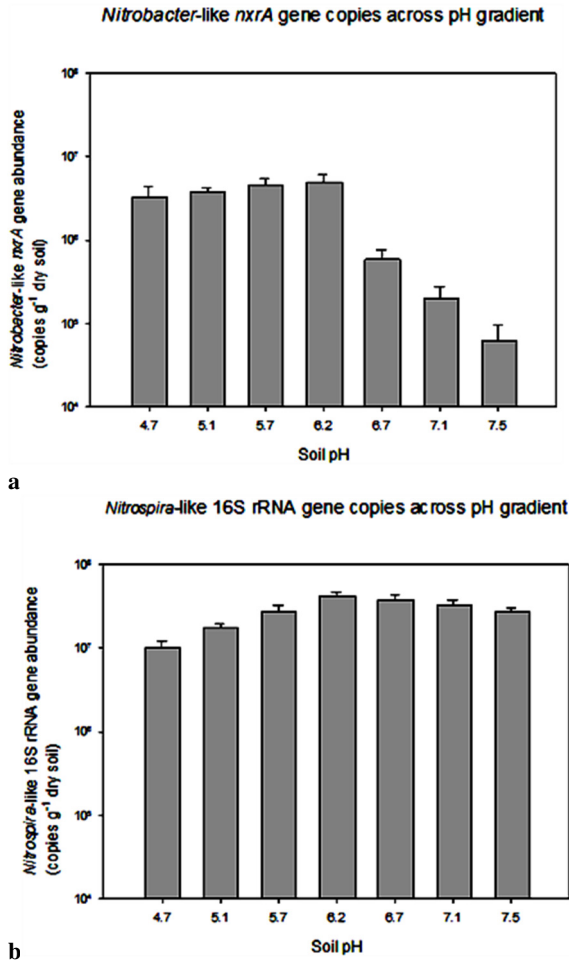


Fig. 1. Effect of pH effects on abundances of *Nitrobacter nxrA* (a) and *Nitrospira* (b) gene abundance ( $\text{g}^{-1}$  dry soil).

**Nitrobacter-like and *Nitrospira*-like NOB gene abundance across pH gradient**

**Nitrifier Community Structures**

Community structures of both *Nitrobacter*-like and *Nitrospira*-like NOB and that of AOB and AOA were characterized by PCR-DGGE analysis of *nxrA* of *Nitrobacter*, *amoA* genes of archaea and 16S rRNA genes of both *Nitrospira*-like NOB and AOB. A variation in DGGE profile with soil pH was observed, which is an indicative of a change in relative abundance of population within *Nitrobacter*-like and *Nitrospira*-like NOB across the pH gradient.

In the *Nitrobacter* community, it was observed that two bands that were dominant at pH 4.7 decreases in relative intensity as the soil pH increased, these band were not detectable at soil plot with high pH (Fig. 2a) in contrast to *Nitrospira* community structure, were it was observed that the relative intensity of the band present at high soil pH 7.1 was decreasing as the soil pH tend to acidic (pH 4.7), and not detectable at this pH (Fig. 2b).

DGGE profile of AOB 16S rRNA genes also show variation with soil pH, several bands were visible at soil pH 4.7 but as the soil pH increases, the relative intensity of these bands keep decreasing until they become undetected at pH 7.5. Also, in soil at pH 7.5 it was observed that the relative intensity of the band present decreases with a decreased in soil pH and was not also detected at soil pH 4.7 (Fig. 2c). DGGE profile of archaea *amoA* genes follow the same pattern as observed in bacterial ammonia oxidisers community structure (Fig. 2d).

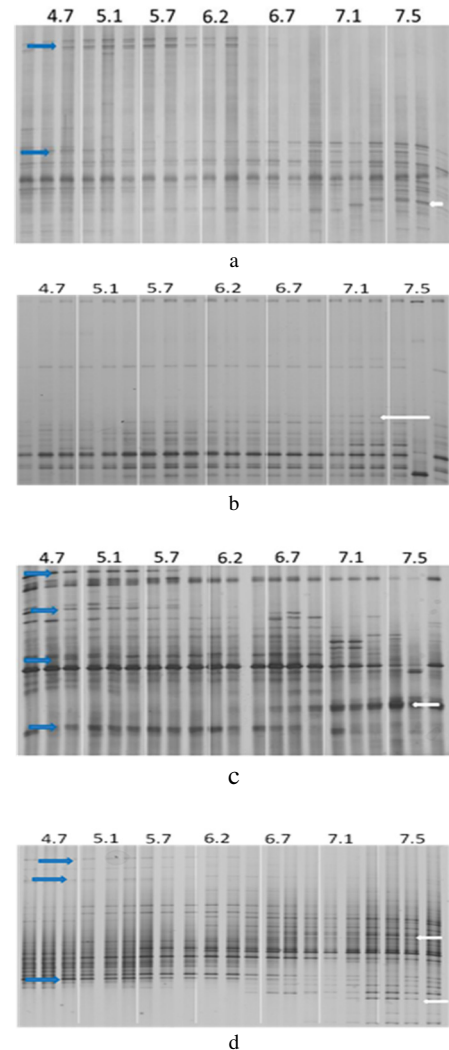


Fig 2. Denaturing gradient gel electrophoresis (DGGE) profiles of *Nitrobacter*-like *nxrA* (a), *Nitrospira*-like 16S rRNA (b), AOB 16S rRNA (c) and AOA *amoA* (d) genes across pH gradients with values in the range 4.5- 7.5 that have been maintained for 46 years. Individual soil samples were represented by its derived profile in each lane. The band position highlighted in blue were those that decreased with an increasing soil pH, while those that increase with and increasing soil pH were highlighted in white.

**Effect of pH on Nitrification Activities of *Nitrobacter*-Like and *Nitrospira*-Like NOB in Microcosm**

The effect of pH on the nitrification activities of *Nitrobacter*-like and *Nitrospira*-like NOB were examined in soil microcosm experiments. Changes in community structures and gene abundance of these two soil groups were also determined. Soil was sampled from high (7.5) and low (4.7) pH plots, respectively.

Nitrite+nitrate concentrations were observed to increase significantly with time in both high and low pH throughout the incubation period for 21 days (Fig. 3).

Nitrite+nitrate concentration increases linearly, with nitrification rate of 0.739 ( $r^2= 0.98$ ) and 0.472 ( $r^2= 0.9$ )  $\text{NO}_2^- + \text{NO}_3^- - \text{N} \mu\text{g g}^{-1}$  dry soil per day for both pH 4.7 and 7.5 soils respectively, as determined by linear regression of nitrite concentration with time. Abundance of *Nitrobacter*-like and *Nitrospira*-like NOB communities in microcosm experiments were assessed by qPCR amplification of *Nitrobacter nxrA* and *Nitrospira* 16S rRNA genes.

The results show that there was no significant increase in *Nitrobacter*-like *nxrA* gene abundance in either pH 4.7 and 7.5 soil during incubation for 21 days (ANOVA, log transformed data,  $P= 0.077$  and 0.58) (Fig. 4a and c). There was also no detectable increase in *Nitrospira*-like 16S rRNA gene abundance in pH 4.5 soil ( $p= 0.665$ ) and pH 7.5 soil sample. (Fig. 4b and d).

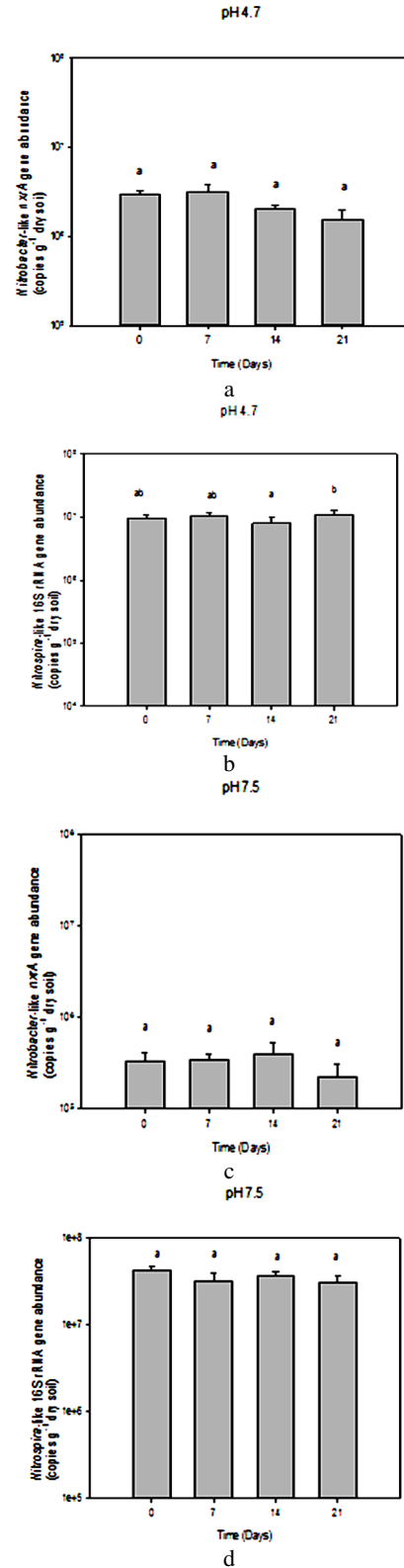
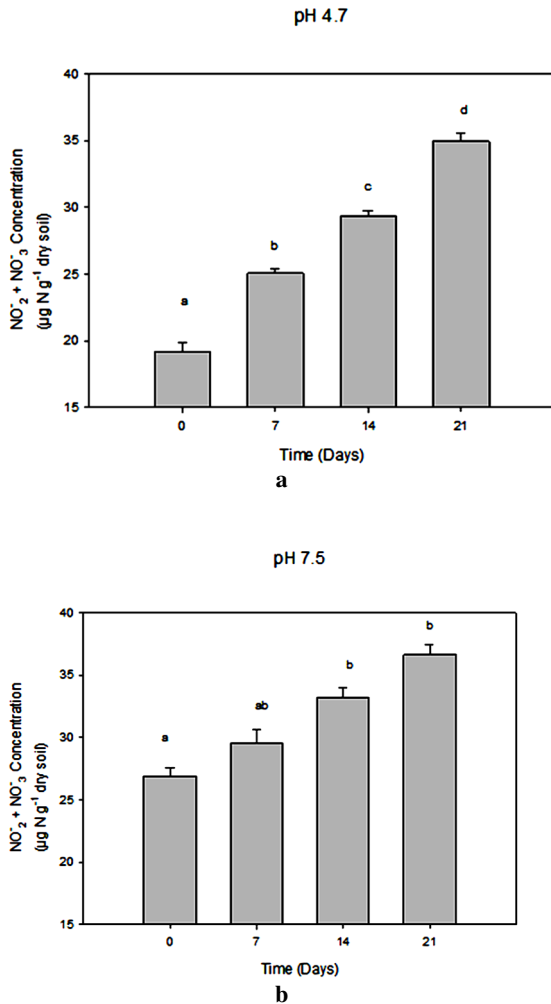


Fig. 3. Change in nitrite+nitrate concentration with time during microcosm incubation of soil at pH 4.7 (a) and 7.5 (b) respectively, different letters above bars indicate significantly different means ( $P < 0.05$ ), means and standard errors were also calculated from triplicate microcosms.

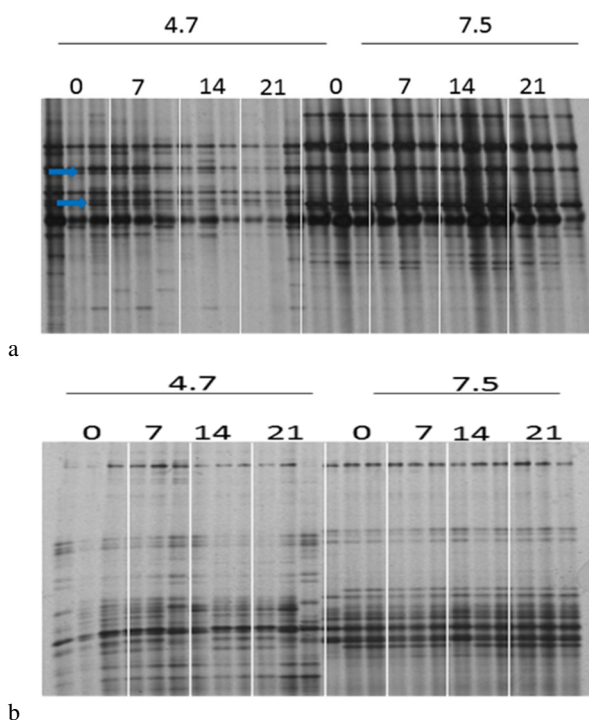
Fig. 4. Changes in abundance of *Nitrobacter nxrA* gene copies (a and c) and *Nitrospira* 16S rRNA gene copies (b and d) during incubation of microcosm containing soil of pH 4.5 and 7.5 for 21 days at 30°C.

### Relationship between Nitrite + Nitrate Concentration and *Nitrobacter*-like and *Nitrospira*-like NOB

A significant weak and negative correlation was observed between nitrite + nitrate concentration and *Nitrobacter*-like *nrxA* gene abundance in pH 4.7 soil ( $p > 0.05$ ,  $r^2 = 0.7757$ ,  $y = 101233x + 5E 06$ ) and no significant relationship in pH 7.5 soil ( $p > 0.05$ ,  $r^2 = 0.2425$ ,  $y = -8574.5x + 599425$ ). There was no significant correlation observed between nitrite + nitrate concentration and *Nitrospira*-like 16S rRNA gene abundance in soil pH 7.5 ( $p > 0.05$ ,  $r^2 = 0.4744$ ,  $y = 84348x + 6E +07$ ), in contrast, a positive correlation was observed for *Nitrospira*-like 16S rRNA gene abundance soil pH 4.7 ( $p < 0.05$ ,  $r^2 = 0.0367$ ,  $y = 35496x + 9E 0+ 6$ ) (Figure not shown).

### *Nitrobacter*-Like and *Nitrospira*-Like NOB Community Structure

The structure of *Nitrobacter*-like and *Nitrospira*-like NOB communities were assessed by DGGE analyses of *nrxA* and 16S rRNA genes fragments amplified from DNA extracted from microcosm soil collected at day (0) and after 21 days of incubation. Comparing the community composition of *Nitrobacter* in pH 4.7 with pH 7.5 soil, it was observed that *Nitrobacter nrxA* profile in pH 4.7 microcosm soil show variation with incubation days, the relative intensity of two band decreased with incubation period from day (0) to day 21 of incubation, whereas in pH 7.5 soil microcosm, no variation was observed in *Nitrobacter nrxA* profile with days of incubation (Fig 5a). In contrast DGGE profiles of *Nitrospira* community in both pH 4.7 and pH 7.5 soil in microcosm showed no variation with days of incubation from day (0) to day 21 were similar (Fig 5b).



**Fig. 5.** DGGE profiles of *Nitrobacter*-like *nrxA* gene (a) and *Nitrospira*-like NOB 16S rRNA gene (b) in both pH 4.5 and 7.5 soil incubated at 30°C for 21 days. Bands highlighted in blue arrow are considered to decrease in relative intensity during the 21-days incubation period.

## DISCUSSION

Studies investigating the ecology of nitrifiers has focused more on AOB [10, 28] and recently AOA [11,14,29], as first step of autotrophic nitrification is assumed to be rate limiting [3,28]. However, relatively little is known about the activities and diversity of soil NOB.

To date only four phylogenetic groups of NOB have been described, with each conforming to a genus, i.e. *Nitrobacter*, *Nitrospira*, *Nitrococcus* and *Nitrospina*. In addition, only *Nitrobacter* species have been isolated from soil to date. Furthermore, *Nitrospira*-like 16S rRNA gene sequences has also been detected in soil [21,22] Autotrophic nitrification is believed to occur in acidic soil, despite the fact that laboratory cultures of isolated ammonia and nitrite oxidising bacteria fail to grow below neutral pH. Previous studies have revealed pH selection for distinct archaeal and bacterial communities in soil that had been maintained for 49 years in pH range 4.5 - 7.5 [9,10,13,26]. This study is aimed to determine whether a similar selection occurred for nitrite oxidising bacteria that perform the second crucial phase in nitrification, within the lineages of *Nitrobacter* and *Nitrospira* encountered in soil.

**Effect of pH on *Nitrobacter* and *Nitrospira* NOB Abundance**  
qPCR quantification of *nrxA* and 16S rRNA genes of both *Nitrobacter*-like and *Nitrospira*-like NOB across plots which has been maintained since 1961 at pH range 4.5 -7.5 showed contrasting pH effects, with *Nitrobacter*-like and *Nitrospira*-like *nrxA* and 16S rRNA abundance decreasing and increasing respectively, as soil acidity increased. *Nitrobacter* and *Nitrospira* gene abundance showed a different pattern with soil pH. A significant decrease in gene abundance of *Nitrobacter*-like *nrxA* genes was observed as soil pH increased, whereas *Nitrospira*-like 16S rRNA gene increased with an increasing soil pH to a maximum at pH 6.2 before decreasing slightly at pH 6.7, 7.1 and 7.5.

Interestingly, pH selection for *Nitrobacter*-like and *Nitrospira*-like NOB observed in all soil pH plots in this study was similar to those observed by [3] for bacterial ammonia oxidiser and archaeal ammonia oxidiser in same soil. These contrasting differences in gene abundance across the pH gradient may reflect different in preferences of *Nitrobacter*-like and *Nitrospira*-like NOB for available nitrite concentration, as it was stated that under low pH, nitrite is available as nitric acid, thus for autotrophic nitrification to continue at low pH, this nitrite must be removed by acid-tolerant NOB of the genus *Nitrobacter* [7,5,30]. In addition, pH is also considered to affects the activities of NOB [32]. Furthermore, the presence of a higher ratio of *Nitrobacter*-like *nrxA* gene versus *Nitrospira*-like 16S rRNA gene abundance occurring at the lowest soil pH is an indication that autotrophic nitrification in acidic soil may be attributed to *Nitrobacter* being the only genus of NOB that has been detected in acidic soils so far [19].

A previous study on the diversity of nitrifiers in geothermal environments provided evidence for high diversity of *Nitrospira* at temperatures ranging from 37 to 58 °C and their ability to grow between pH 7.0 and 7.8 corresponding to preferences of alkali-tolerant organisms which are more active between pH 7.0 – 7.8. This result is in accordance with our result, where *Nitrospira* genes were observed to be more abundant in alkaline pH between 6.2 to 7.5 soil plots. The first isolated acidophilic chemoautotrophic *Nitrobacter* was in soil, which shows optimum growth at pH range between 4.6 – 5.5 °C [30]. Thus, this is also similar to those observed for *Nitrobacter* gene in this work. A

recent study on the effects of pH on NOB activity showed that NOB activity is optimum at pH 7.9 and reduced by 20% at pH 6.5 and 9 [33] and completely inhibited at pH values below 6.5 [32]. This is in contrast to these results which showed more abundant of *Nitrobacter*-like NOB genes at low pH values while *Nitrospira*-like NOB gene abundant are at maximum at pH 6.2, thus, indicating that the activities of NOB is possible in both acidic pH 4.7 and neutral pH 6.2.

#### Effects of soil pH on nitrifier community structures

DGGE analysis of *Nitrobacter*-like *nxrA* and *Nitrospira*-like 16S rRNA gene profiles across pH gradient indicate that the genetic structure of both *Nitrobacter*-like and *Nitrospira*-like NOB communities are influence by pH across the gradient. As previously observed [3], archaeal and bacterial ammonia oxidizers activity and distribution in this soil is influenced by soil pH. Previous studies have revealed that soil pH leads to selection for different archaeal and bacterial communities [3,10,13]. This result match with these work, which shows that *Nitrobacter*-like *nxrA* band were dominant at pH 4.7 but decreases in relative intensity as the soil pH increased and not detected at soil plot with high pH whereas *Nitrospira* community structure were present at high soil pH 7.1 but decreasing as the soil pH becomes more acidic, thus this variation with pH suggest adaptation and selection of a particular phylogenetic groups in acid soil [4]. This is also in agreement with [34] showed that bacterial community structure and diversity is determined by pH. In contrast, it was reported that *Nitrospira*-like and not *Nitrobacter*-like community composition and diversity change following long term N fertilization of agricultural soil [22].

However, nitrogen fertilization causes a decrease in soil pH, thus changes in pH across the gradient is responsible for the pronounced shift observed in bacterial community composition across these gradient, the relative abundance of *Nitrobacter*-like and not *Nitrospira*-like NOB would be expected to increase at low pH as observed in our results. The bacterial and archaeal ammonia oxidiser community structure investigated by [3,25] were also revisited to determine whether the influence of pH on the community composition of these organisms persist with time. Interestingly, bacterial and archaeal ammonia oxidiser DGGE profiles showed variation with soil pH, demonstrating changes in the relative abundance of both bacterial and archaeal ammonia oxidiser across the gradient. Thus, archaea *amoA* gene DGGE profile showed

Several bands at soil pH 4.7 which relative intensity decreased with increasing soil pH until they become invisible at pH 7.5, and at pH 7.5 the relative intensity of the band present decreases with a decreased in soil pH and became undetectable at soil at pH 4.7. DGGE profile of bacterial ammonia oxidiser follow the same pattern as observed in archaeal ammonia oxidisers community structure. Bacterial and archaeal ammonia oxidiser community structure were similar to those observed before [3], who also found that distinct archaeal and bacterial communities composition is influenced by pH, as observed here in all soil pH plots.

#### Effect of pH on nitrification activities of *Nitrobacter*-Like and *Nitrospira*-Like NOB in soil microcosm

Nitrification activity of both *Nitrospira*-like and *Nitrobacter*-like NOB in acidic and neutral pH soil was investigated in a microcosm experiment incubated at 30 °C for 21 days. The abundance and community structures of both organisms were also examined in high and low pH soil (4.7 and 7.5) microcosm. The result show that no detectable increase in abundance of both *Nitrobacter* and *Nitrospira* genes either in pH 4.7 and 7.5 soil

during incubation for 21 days. The low activities of *Nitrobacter* and *Nitrospira* in this soil may be attributed to incubation temperature used in the microcosm study, as temperature plays a significant role in community changes, thus, relatively low temperature 24 – 25 °C were favourable for *Nitrobacter* while *Nitrospira* were more adapted to high temperature. In addition, anoxic conditions are known to inhibit nitrite oxidation activity, however, we have no direct evidence that during our microcosm experiments oxygen limitation occurred as aerobic conditions were maintained during incubation by removing seal and venting for 10 minutes every two days.

*Nitrospira*-like community structure showed no variation in both low and high pH soil microcosm after incubation for 21 days and there was no detectable increase in *Nitrospira*-like 16S rRNA gene abundance in either soil. In contrast, the composition of *Nitrobacter*-like NOB community in pH 4.7 soil were observed to varied with incubation days while at higher pH soil *Nitrobacter*-like community composition remained unchanged throughout the incubation period, indicating no significant increase in *Nitrobacter*-like gene abundance in pH 7.5.

#### Relationship between Nitrite + Nitrate concentration and *Nitrobacter*-Like and *Nitrospira*-Like NOB gene abundance

Nitrite + nitrate concentration was strongly correlated with abundance of *Nitrospira*-like but not *Nitrobacter*-like NOB. This is in contrast to [21] showed a strong positive correction between potential nitrite oxidation and *Nitrobacter*-like NOB abundances and report a weak and negative relation for *Nitrospira*-like NOB abundances. Similarly, provide evidence for strong correction between nitrification and *Nitrobacter* abundance in sterile soil microcosm.

The differences in relative contribution of *Nitrospira* and *Nitrobacter*-like NOB to nitrification in soil pH 4.7 and 7.5 observed in this study may be attributed to nitrite availability, as there is a strong evidence for preference of *Nitrospira*-like NOB to low nitrite concentration [35]. Our result, therefore, suggests that *Nitrospira*-like rather than *Nitrobacter*-like NOB play a major functional role in soil with low nitrite concentration.

#### CONCLUSION

In conclusion, this study revealed that different *Nitrobacter*-like and *Nitrospira*-like NOB are selected in soil of different pH. Thus, quantification of gene copies indicates that the differences in abundance and community structures is a reflection of different activity of NOB. The finding suggested that *Nitrobacter*-like NOB rather than *Nitrospira*-like NOB are adapted to acidic pH. In addition, the study also confirmed soil pH as the major potential driver determining the distribution and abundance of both *Nitrobacter*-like and *Nitrospira*-like NOB in soil. This study is the first to investigate the effect of soil pH on nitrite oxidizing bacteria. Furthermore, the total diversity of NOB in soil is yet to be fully elucidated thus, remains an area ripe for further research.

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