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| **\*Title:** | Adaptive responses of comammox *Nitrospira* and canonical ammonia oxidizers to long-term fertilizations: implications for the relative contributions of different ammonia oxidizers to soil nitrogen cycling |
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| **\*CATEGORY:** | Agricultural and Biological Sciences |

**Data Article**

**Title**: Soil properties, canonical ammonia oxidizers and comammox data of long-term gradients application of fertilizers

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**Abstract**

In this data article, we provide the scientific and original data on soil properties and soil potential ammonia oxidation (PAO) and potential nitrite oxidation (PNO) data. The original data of the abundances of ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA) and comammox using quantitative PCR were also reported. In addition, we preformed terminal restriction fragment length polymorphism (T-RFLP) to investigate the compositions of comammox, and the original data were also described here. These data are the foundation of the analyses and results in the article “Adaptive responses of comammox *Nitrospira* and canonical ammonia oxidizers to long-term fertilizations: implications for the relative contributions of different ammonia oxidizers to soil nitrogen cycling”.

**Specifications Table**

|  |  |
| --- | --- |
| Subject area | Biology |
| More specific subject area | Agricultural and Biological Sciences |
| Type of data | Table |
| How data was acquired | Surveys, quantitative PCR, terminal restriction fragment length polymorphism |
| Data format | Raw |
| Experimental factors | PCR reactions were pooled and purified using the EZNA Cycle-Pure Kit (Omega Bio-tek Inc, Doraville, GA, USA) |
| Experimental features | Soil samples were collected from a long –term field under gradient of nutrients input |
| Data source location | Qianyanzhou Ecological Station, Jiangxi province, China (26°44’ N, 115°03’ E) |
| Data accessibility | Data are with this article. The 58 sequences obtained in this study were deposited in GenBank under the Accession Numbers MK167293 to MK167350. |
| Related research article | None |

**Value of the Data**

1. The data were collected from an experimental field that has undergone 19 years of fertilization using varying amounts of N, phosphorus (P) and potassium (K) fertilizers.
2. Less is known about the response of new discovery of complete ammonia oxidizers (comammox) to different fertilizers. The data may serve as a benchmark for future studies of comammox and their response to fertilizations in arable soils.

**1. Data**

This article includes the raw data on the effects of gradient of nutrients input on changes in soil properties, soil potential ammonia oxidation (PAO) and potential nitrite oxidation (PNO), amoA gene abundances of AOB, AOA and comammox, relative abundance of different terminal restriction fragments of comammox (Table 1, Table 2, Table 3, Table 4). This long-term fertilization experiment was established in 1998 with randomized block design, including 9 treatments with 3 replicates. The data of these three replicates that used in the main manuscript were shown here.

Table 1. Soil properties under different fertilization treatments

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Treatments | pH | SOM b  (g Kg-1) | TN  (g Kg-1) | AN  (mg Kg-1) | AP  (mg Kg-1) | AK  (mg Kg-1) | TP  (g Kg-1) |
| CK-1a | 5.72 | 13.809 | 0.811125 | 73.81237 | 7.421793 | 17.1046 | 0.12 |
| CK-2 | 5.90 | 13.10134 | 1.013251 | 73.7647 | 4.468438 | 18.84647 | 0.11 |
| CK-3 | 5.80 | 13.4077 | 0.90901 | 69.1171 | 6.393372 | 15.94808 | 0.12 |
| N1P1K1-1 | 5.91 | 18.57478 | 1.321368 | 105.0752 | 29.39537 | 25.86833 | 0.24 |
| N1P1K1-2 | 6.44 | 22.93218 | 1.218479 | 112.5818 | 37.36668 | 24.03449 | 0.21 |
| N1P1K1-3 | 6.01 | 19.84053 | 1.009511 | 97.7449 | 25.03587 | 27.96346 | 0.24 |
| N2P2K2-1 | 6.42 | 18.74532 | 1.016183 | 101.7199 | 41.35864 | 57.05866 | 0.26 |
| N2P2K2-2 | 6.30 | 21.9491 | 1.01852 | 111.9638 | 44.36672 | 53.98154 | 0.25 |
| N2P2K2-3 | 6.22 | 23.32672 | 1.110719 | 116.88 | 50.46705 | 62.57894 | 0.29 |
| N3P3K3-1 | 6.63 | 21.01905 | 1.117602 | 107.9888 | 56.96384 | 112.4714 | 0.56 |
| N3P3K3-2 | 6.49 | 20.03731 | 1.111724 | 109.6281 | 62.22286 | 111.5261 | 0.50 |
| N3P3K3-3 | 6.53 | 22.14261 | 1.11245 | 118.1665 | 64.48839 | 112.9137 | 0.37 |
| N4P4K4-1 | 7.05 | 22.23587 | 1.320251 | 113.8584 | 96.02285 | 209.666 | 0.40 |
| N4P4K4-2 | 7.06 | 20.78592 | 1.215811 | 107.6885 | 64.48866 | 179.9907 | 0.52 |
| N4P4K4-3 | 6.85 | 23.89993 | 1.214284 | 125.9698 | 74.0713 | 188.5175 | 0.60 |
| N3P1K1-1 | 5.81 | 20.82077 | 1.319337 | 113.0408 | 21.96189 | 27.22909 | 0.21 |
| N3P1K1-2 | 6.00 | 20.01873 | 1.110693 | 113.9369 | 21.32531 | 21.0123 | 0.18 |
| N3P1K1-3 | 6.19 | 23.0378 | 1.012348 | 104.6525 | 19.07264 | 22.25141 | 0.25 |
| N1P3K1-1 | 6.33 | 23.4807 | 1.219692 | 116.9115 | 62.20429 | 30.04508 | 0.47 |
| N1P3K1-2 | 6.39 | 20.56123 | 1.010718 | 110.5175 | 65.59557 | 22.08418 | 0.67 |
| N1P3K1-3 | 6.52 | 20.07395 | 1.215007 | 101.7204 | 54.77658 | 25.54553 | 0.48 |
| N1P1K3-1 | 6.24 | 19.58544 | 0.912894 | 102.6418 | 26.27107 | 103.4867 | 0.25 |
| N1P1K3-2 | 6.24 | 17.31314 | 1.059754 | 100.6625 | 31.04573 | 82.33276 | 0.23 |
| N1P1K3-3 | 6.21 | 22.87785 | 1.215592 | 111.7251 | 21.7591 | 100.2863 | 0.24 |
| OM-1 | 5.86 | 27.87876 | 1.322155 | 150.3026 | 90.56762 | 27.90764 | 0.36 |
| OM-2 | 6.05 | 28.47503 | 1.733248 | 156.6123 | 102.8224 | 33.79835 | 0.53 |
| OM-3 | 5.88 | 32.82548 | 1.519173 | 155.5714 | 123.0024 | 27.43626 | 0.33 |

aThe symbol and nutrient input of each treatments is described in the section Experimental Design, Materials, and Methods

*b* Abbreviations: SOM stands for soil organic matter, TN stands for total N, TP stands for total P, AN stands for available N, AP stands for available P, AK stands for available K.

Table 2. Soil potential ammonia oxidation (PAO) and potential nitrite oxidation (PNO) under different fertilization treatments

|  |  |  |
| --- | --- | --- |
| Treatments | PAO b  (ng N g-1 h-1) | PNO  (ng N g-1 h-1) |
| CK-1 a | 122.3583 | 106.7651 |
| CK-2 | 191.4204 | 140.8644 |
| CK-3 | 206.8679 | 136.6013 |
| N1P1K1-1 | 306.3274 | 677.1239 |
| N1P1K1-2 | 249.2869 | 510.9676 |
| N1P1K1-3 | 304.6876 | 470.0125 |
| N2P2K2-1 | 394.2874 | 708.4369 |
| N2P2K2-2 | 340.6047 | 753.4729 |
| N2P2K2-3 | 373.6551 | 908.214 |
| N3P3K3-1 | 347.7555 | 1056.41 |
| N3P3K3-2 | 406.1474 | 911.8321 |
| N3P3K3-3 | 422.2506 | 968.2576 |
| N4P4K4-1 | 819.7013 | 1132.4831 |
| N4P4K4-2 | 995.0377 | 1996.979 |
| N4P4K4-3 | 1055.772 | 926.9188 |
| N3P1K1-1 | 230.3405 | 801.6073 |
| N3P1K1-2 | 281.6942 | 594.2694 |
| N3P1K1-3 | 260.8737 | 507.4315 |
| N1P3K1-1 | 290.6075 | 510.5613 |
| N1P3K1-2 | 350.639 | 285.621 |
| N1P3K1-3 | 258.9394 | 355.5594 |
| N1P1K3-1 | 182.8122 | 694.0362 |
| N1P1K3-2 | 225.9367 | 844.5495 |
| N1P1K3-3 | 216.6269 | 1010.7652 |
| OM-1 | 361.8968 | 1025.3587 |
| OM-2 | 342.3242 | 1090.1269 |
| OM-3 | 247.265 | 1058.7542 |

aThe symbol and nutrient input of each treatments is described in the section Experimental Design, Materials, and Methods

bAbbreviations: PAO stands for potential ammonia oxidation, PNO stands for potential nitrite oxidation.

Table 3. Abundances of canonical ammonia oxidizers and comammox under different fertilization treatments

|  |  |  |  |
| --- | --- | --- | --- |
| Treatments | AOB b  (*amoA* gene copies per gram soil) | AOA  (*amoA* gene copies per gram soil) | Comammox  (*amoA* gene copies per gram soil) |
| CK-1 a | 7222321 | 49377211 | 141610 |
| CK-2 | 5769325 | 63444734 | 93859 |
| CK-3 | 5744726 | 58814132 | 143985 |
| N1P1K1-1 | 10999222 | 95139345 | 488465 |
| N1P1K1-2 | 11733530 | 74044214 | 446611 |
| N1P1K1-3 | 9514649 | 69858142 | 387412 |
| N2P2K2-1 | 10463193 | 68840071 | 435880 |
| N2P2K2-2 | 7832099 | 53889496 | 249204 |
| N2P2K2-3 | 7805413 | 65321293 | 290387 |
| N3P3K3-1 | 9458379 | 53615790 | 371999 |
| N3P3K3-2 | 10041312 | 51802641 | 323896 |
| N3P3K3-3 | 10551603 | 41755997 | 311847 |
| N4P4K4-1 | 15707065 | 70740425 | 515380 |
| N4P4K4-2 | 13767875 | 69250500 | 164706 |
| N4P4K4-3 | 11828686 | 23897738 | 345697 |
| N3P1K1-1 | 10801170 | 95416665 | 632017 |
| N3P1K1-2 | 12906293 | 124763851 | 345474 |
| N3P1K1-3 | 11071418 | 139377828 | 663476 |
| N1P3K1-1 | 10227751 | 64797064 | 473147 |
| N1P3K1-2 | 8585580 | 50119367 | 252913 |
| N1P3K1-3 | 9117555 | 49802923 | 362501 |
| N1P1K3-1 | 10963039 | 54715774 | 273839 |
| N1P1K3-2 | 8103207 | 60028809 | 368442 |
| N1P1K3-3 | 6782438 | 73430418 | 320450 |
| OM-1 | 12440001 | 93274409 | 314902 |
| OM-2 | 11766244 | 120507982 | 473534 |
| OM-3 | 13720542 | 55274375 | 252277 |

aThe symbol and nutrient input of each treatments is described in the section Experimental Design, Materials, and Methods

bAbbreviations: AOB stands for ammonia oxidizing bacteria, AOA stands for ammonia oxidizing archaea.

Table 4. Relative abundance (%) of individual terminal restriction fragment of comammox under different fertilization treatments

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Treatments | 43 bp | 47 bp | 53 bp | 73 bp | 74 bp | 78 bp | 102 bp | 118 bp | 121 bp | 141 bp | 148 bp | 193 bp | 198 bp |
| CK-1 a | 0 | 0 | 0 | 0 | 0 | 1.82 | 0 | 0 | 0 | 0 | 0 | 7.9 | 90.27 |
| CK-2 | 0 | 2.36 | 0 | 1.04 | 0 | 5.4 | 0 | 0.87 | 2.93 | 0 | 0 | 0 | 87.4 |
| CK-3 | 7.18 | 5.38 | 1.55 | 1.62 | 0 | 2.35 | 0 | 0 | 1.37 | 0 | 0 | 0 | 80.56 |
| N1P1K1-1 | 0 | 0 | 0 | 9.1 | 0 | 4.97 | 0 | 3.23 | 16.91 | 3.72 | 0 | 0 | 62.07 |
| N1P1K1-2 | 0.92 | 0 | 0.59 | 12.66 | 0 | 3.03 | 0 | 0.91 | 3.84 | 2.16 | 0.76 | 0 | 75.14 |
| N1P1K1-3 | 0 | 0 | 0 | 12.77 | 0 | 6.12 | 0 | 3.43 | 17.7 | 5.48 | 0 | 0 | 54.5 |
| N2P2K2-1 | 0 | 1.88 | 3.66 | 15.89 | 1.39 | 2.13 | 5.26 | 2.7 | 0 | 0 | 0 | 0 | 67.09 |
| N2P2K2-2 | 2.04 | 0 | 0 | 16.26 | 0 | 3.92 | 0 | 14.41 | 0 | 5.31 | 0 | 0 | 58.06 |
| N2P2K2-3 | 0 | 0 | 0.64 | 28.03 | 1 | 4.49 | 0 | 2.15 | 5.92 | 5.74 | 0.38 | 0 | 51.65 |
| N3P3K3-1 | 0 | 0 | 0 | 17.59 | 0 | 1.58 | 0 | 7.85 | 0 | 1.99 | 0 | 0 | 70.98 |
| N3P3K3-2 | 0 | 0 | 0.77 | 28.73 | 0 | 2.9 | 0 | 9.38 | 0 | 5.57 | 0 | 0 | 52.65 |
| N3P3K3-3 | 0 | 0 | 0 | 23.1 | 0 | 2.58 | 0 | 10.9 | 0 | 1.62 | 0 | 0 | 61.8 |
| N4P4K4-1 | 0 | 0 | 1.26 | 32.54 | 0 | 4.82 | 0 | 8.81 | 0 | 2.7 | 0 | 0 | 49.87 |
| N4P4K4-2 | 0 | 0 | 0 | 28.08 | 0 | 5.29 | 0.52 | 3.2 | 0 | 6.74 | 0 | 0 | 56.17 |
| N4P4K4-3 | 0 | 0 | 0 | 19.26 | 0 | 2.5 | 0 | 5.43 | 0 | 8.48 | 0 | 0 | 64.34 |
| N3P1K1-1 | 0 | 0 | 0 | 16.88 | 0 | 5.17 | 0 | 3.38 | 11.49 | 4.8 | 0 | 0 | 58.27 |
| N3P1K1-2 | 0 | 1.09 | 0 | 9.41 | 0 | 2.45 | 0 | 1.9 | 9.43 | 3.26 | 0 | 0 | 72.46 |
| N3P1K1-3 | 1.13 | 0.7 | 0.98 | 42.13 | 0 | 5.38 | 0 | 0.69 | 2.37 | 2.41 | 0 | 0 | 44.2 |
| N1P3K1-1 | 0 | 0 | 0 | 25.99 | 0 | 3.62 | 0 | 13.65 | 0 | 5.22 | 0 | 2.09 | 49.43 |
| N1P3K1-2 | 1.67 | 0.48 | 0.69 | 14.23 | 0 | 1.53 | 0 | 3.17 | 0 | 2.4 | 0 | 0 | 75.82 |
| N1P3K1-3 | 4.25 | 0.99 | 0.89 | 20.98 | 0 | 4.34 | 0 | 2.02 | 7.14 | 3.21 | 0 | 0 | 56.17 |
| N1P1K3-1 | 0 | 0 | 0.76 | 16.32 | 3.78 | 4.35 | 16.13 | 3.28 | 1.15 | 0 | 0 | 0 | 54.23 |
| N1P1K3-2 | 2.86 | 1.48 | 0 | 11.45 | 0 | 2.04 | 0 | 1.47 | 9.99 | 2.18 | 0 | 0 | 68.54 |
| N1P1K3-3 | 0 | 0.68 | 1.02 | 24.32 | 0 | 1.38 | 0 | 3.46 | 0 | 0.71 | 0 | 0 | 68.43 |
| OM-1 | 0 | 0 | 0 | 3.71 | 0 | 7.65 | 0 | 3.63 | 17.97 | 0 | 0 | 0 | 67.03 |
| OM-2 | 1.24 | 0 | 0 | 8.03 | 0 | 3.55 | 0 | 2.35 | 9.26 | 2.56 | 0 | 0 | 73.01 |
| OM-3 | 0 | 0.80 | 0 | 2.55 | 0 | 4.03 | 0 | 2.94 | 16.3 | 2.73 | 0 | 0 | 70.64 |

aThe symbol and nutrient input of each treatments is described in the section Experimental Design, Materials, and Methods

**2. Experimental Design, Materials, and Methods**

*2.1. Site description*

The experimental site is situated at Qianyanzhou Ecological Station, Jiangxi province, China (26°44’ N, 115°03’ E) and is a double-season rice system. The site has a subtropical monsoon climate in a hilly region and the soil type is waterloggogenic paddy soil. The average annual temperature is 16.7 °C. Annual precipitation varies from 1200 to 1900 mm.

*2.2. Experimental design and* *soil sampling*

The long-term fertilization experiment was established in 1998 with randomized block design, including 9 treatments with 3 replicates. Each replicate plot was 3×5 m, and was isolated by concrete walls (50 cm depth and 15 cm above the soil surface). The 9 experimental treatments were: (1) CK, no fertilization control; (2) N1P1K1, 228 kg ha-1 urea (46 % N), 461 kg ha-1 fused calcium/magnesium phosphate (13 % P2O5) and 175 kg ha-1 potassium chloride (60 % K2O) were applied for the early rice season, and 261 kg ha-1 urea, 577 kg ha-1 fused calcium/magnesium phosphate and 200 kg ha-1 potassium chloride were applied for the late rice season; (3) N2P2K2, where 50 % larger amounts of N, P and K were applied when compared to N1P1K1; (4) N3P3K3, where 100 % larger amounts of N, P and K were applied when compared to N1P1K1; (5) N4P4K4, where 200 % larger amounts of N, P and K were applied when compared to N1P1K1; (6) N3P1K1, applied the same amounts of P and K but 100 % more N than N1P1K1; (7) N1P3K1, applied same amounts of N and K but 100 % more P than N1P1K1; (8) N1P1K3, applied same amounts of N and P but 100 % more K than N1P1K1; (9) OM, applied 19,067 kg ha-1 of organic manure (0.55 % N, 0.95 % P2O5 and 1.1 % K2O, 10.2 % organic C). OM had the same amount of N as N1P1K1. Organic manure fertilizer was made of composted pig manure and rice straw; the composting-process was conducted at high temperatures and a good organic fertilizer was obtained after sterilization and deodorization ([Dong et al., 2012](#_ENREF_8)). The details of the experimental design and the amounts of N, P, and K are shown in Table 1. All the organic fertilizer and 60 % of inorganic fertilizers were applied as base fertilizers before sowing; the remaining inorganic fertilizers were applied as top-dressing seven days after the rice was transplanted.

Soil samples were collected in October 2017 after the late rice was harvested. Five soil cores at a depth of 0-20 cm were collected from each plot. After mixing thoroughly and being passed through a 2-mm sieve, the fresh soil was used to measure available N (AN), potential ammonia oxidation (PAO) and potential nitrite oxidation (PNO), and extract soil DNA. A subsample of soil was air-dried to measure soil pH, SOM (soil organic matter), total N (TN), total P (TP), available P (AP) and available K (AK).

*2.3. Assays for soil properties, PAO and PNO*

Soil pH was determined with a soil-to-water ratio of 1:2.5. Soil AN was determined by titrating after alkaline hydrolysis. SOM and TN contents were measured using a Vario-MaxN/CN elemental analyzer (Elementar Analysensysteme Gmb H, Hanau, Germany). Soil TP and AP were measured by the molybdenum-blue method ([Olsen, 1954](#_ENREF_28)). Soil AK was measured using a flame atomic absorption spectrophotometer ([Brown, 1998](#_ENREF_2)).

Soil PAO was measured using the chlorate inhibition method ([Kurola et al., 2005](#_ENREF_23)) with minor modifications. Briefly, 5.0 g of fresh soil was agitated in 20 mL of phosphate buffer solution (NaCl, 8.0 g L-1; KCl, 0.2 g L-1; Na2HPO4, 0.2 g L-1; NaH2PO4, 0.2 g L-1; pH 7.4) with 1 mM (NH4)2SO4. To inhibit nitrite oxidation, a final concentration of 10 mM potassium chlorate was added to the tube. After incubating the suspension for 24 hours on a rotary shaker at 25 °C and 170 rpm, NO2--N was extracted by 5 mL of 2 M KCl and determined spectrophotometrically at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride.

Soil PNO was measured using the method described by [Wertz et al. (2007](#_ENREF_44). Briefly, 5.0 g of fresh soil was incubated with 30 mL of NaNO2 solution (10 μg of N-NO2- g-1 dry soil), after incubating the suspension for 15 hours on a rotary shaker at 25 °C and 170 rpm, NO2--N was extracted and determined using the same method as PAO.

*2.4. Soil DNA extraction and measurement of AOB, AOA and comammox Nitrospira abundances by* *quantitative PCR*

DNA was extracted from each plot using 0.25 g of fresh soil with MoBio PowerSoilDNA Isolation Kits (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Quantitative PCR reactions were performed using the SYBR Premix Ex TaqTM (Perfect Real Time) kit (TaKaRa Biotechnology Co., Dalian, China). The 25 μL final volume PCR reaction contained 12.5 μL of SYBR Premix Ex TaqTM (2×, Takara), 0.5 μL ROX Reference Dye II (50×, TaKaRa), 10 μL dd H2O, 0.5 μL (5 μM) of each primer and 1 μL DNA template. The AOB *amoA* gene primers were *amoA*-1F (5’-GGGGTTTCTACTGGTGGT-3’) and *amoA*-2R (5’-CCCCTCKGSAAAGCCTTCTTC-3’) and yielded a fragment of 491 bp in length ([McTavish et al., 1993](#_ENREF_25)). The AOA *amoA* gene primers were Arch-*amoA*F (5’-STAATGGTCTGGCTTAGACG-3’) and Arch-*amoA*R (5’-GCGGCCATCCATCTGTATGT-3’) and yielded a fragment of 635 bp in length ([Francis et al., 2005](#_ENREF_12)). The comammox *Nitrospira amoA* gene primers were Ntsp-*amoA* 162F (5’-GGATTTCTGGNTSGATTGGA-3’) and Ntsp-*amoA* 359R (5’-WAGTTNGACCACCASTACCA-3’) and yielded a fragment of 198 bp in length.

Thermal cycling was as follows: an initial activation step at 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C 34 s for AOB and AOA, or 60.5 °C 34 s for comammox *Nitrospira*. PCR products of *amoA* genes fragments from soil samples were inserted into PMD18-T plasmids. After sequencing and confirming that sequence using BLAST in GenBank on the NCBI’s homepage (<http://blast.ncbi.nlm.nih.gov/Blast>), the proper gene inserts were chosen to serve as standards. The concentrations of the standard plasmids were measured using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Standard curves were developed by serially diluting plasmids to the final concentrations of 107 to 101 gene copies μL-1. A negative control was run with water as the template. The efficiencies of quantitative PCR were 105 %, 91 %, and 84 % for AOB, AOA and comammox *Nitrospira,* respectively, and the R2 values of the standard curves were higher than 0.99. The possibility of PCR inhibition was examined by serial dilution, and no severe inhibition was found.

*2.5. Terminal restriction fragment length polymorphism (T-RFLP) analysis of* *comammox Nitrospira amoA gene*

T-RFLP analysis was used to analyze community compositions of comammox *Nitrospira amoA* gene. The PCR reaction was performed in a 25 μL volume containing 10.5 μL of ddH2O, 12.5 μL of Premix Taq(2×) Version 2.0 (TaKaRa), 1 μL DNA template, 0.5 μL forward primer (5 μM) Ntsp-*amoA* 162F with 6-FAM (6-carboxyfluorescein) labeled at the 5′ end, 0.5 μL reverse primer (5 μM) Ntsp-*amoA* 359R. The PCR protocol for comammox *Nitrospira amoA* gene was replicated three times following the programs: 3 min at 95 °C for initial denaturing, followed by 33 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 25 s with the final extension for 7 min at 72 °C. After amplification, the triplicate PCR reactions were pooled and purified using the EZNA Cycle-Pure Kit (Omega Bio-tek Inc, Doraville, GA, USA). The same amount of PCR purification product (200 ng) for each sample was digested with 10 units of restriction enzyme *HhaI* (TaKaRa) at 37 °C for 6 h, and the tubes were vortexed every 30 min to ensure complete digestion. After digestion, samples were denatured at 80 °C for 10 min. Capillary electrophoresis was used to separate samples and the precise lengths of the terminal restriction fragments (T-RFs) were estimated. GeneMapper 4.0 software (Applied Biosystems) was used to generate the T-RFLP profiles, peak heights less than 100 fluorescence units were removed from the future analyses.

Lengths of T-RFs less than 40 bp were removed from future analyses. 198 bp T-RF was treated as having no restriction enzyme cutting site and remained for future analysis. The relative abundances of the T-RFs were calculated as the percentages of total peak area in the T-RFLP profile.

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