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Method Article

The application of ^{15}N isotope tracer in differentiating denitrification, anammox and DNRA during anammox start-up by adding calcium nitrate

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A B S T R A C T

Denitrification, anaerobic ammonium oxidation (anammox) and dissimilatory reduction of nitrate to ammonium (DNRA) are important forms of nitrogen transformation process. The addition of calcium nitrate induces the coupling of denitrification, anammox and DNRA in the malodorous sediment, which accelerates the start-up of anammox process. However, conventional detection methods are difficult to differentiate the above-mentioned nitrogen transformation processes. A modified ^{15}N isotope tracer technology was used to quantitatively differentiate each N-removal contribution of denitrification, anammox and DNRA in this research, which is of great significance for ascertaining the coupling relationship among denitrification, anammox and DNRA induced by calcium nitrate.

- A modified ^{15}N isotope tracer technology was used to quantitatively differentiate denitrification, anammox and DNRA.
- ^{15}N isotope tracer results indicated that the contribution of anammox to total nitrogen increased by 20% approximately.

A R T I C L E I N F O

Method name: ^{15}N isotope tracer for determinating the rate of nitrate dissimilatory reduction process induced by calcium nitrate
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*Method details

Detailed steps for ^{15}N isotope tracing

Measurement and calculation of denitrification and anammox rates

Step 1: sample pretreatment. The ^{15}N isotope trace experiments were modified according to the pervious method [1]. The specific pretreatment steps were as follows: 50 mL of sediment sample which comes from one typical malodorous river named Gongye river (Shanghai, China) were collected in 100 mL conical bottle and purged with 5–10 min helium-stripping to remove oxygen. Then, the conical bottle was cultured at ambient temperature for 48 h to remove the residual nitrite and nitrate by the internal biological nitrogen removal process. A portion of the 50 mL sediment sample was weighed and dried to calculate the water content. After 48 h, about 1 mL of sediment sample was taken out and put into 12 mL glass vials (Exetainer, Labco, High Wycombe, Buckinghamshire, UK) and obtained the weight of sediment, and then 9 mL of deionized water was added to make a homogeneous slurry [2]. The purpose of sample pretreatment was to exclude other interference factors before determination.

Step 2: setting of ^{15}N isotope experimental group

Afterward, the nitrogen-containing chemicals with ^{15}N isotope marker were injected into the slurry to maintain a total concentration of 100 μmol/L ^{15}N in each vial. The vials were divided into three treatment groups: (1) blank (without ^{15}N isotope compound); (2) $^{15}NH_4^+$ (It is used to detect whether the residual nitrite and nitrate are completely consumed); and (3) $^{15}NO_2^-$/$^{15}NO_3^-$ (It is used to determine the rates of denitrification and anammox. Due to the high level of $^{14}NH_4^+$ in the sediment, there is no need to add $^{14}NH_4^+$ in this experiment). These treatments were continuously incubated at ambient temperature for 8 h, and then 200 μL of 50% $ZnCl_2$ saturated solution was injected to terminate the biological nitrogen removal reaction in sediment. After fully shaking and clarification, the supernatant of the glass vial was measured by membrane inlet mass spectrometry (MIMS) after filtered through a 0.45 μm filter membrane. The obtained data of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ production were used to calculate the rates of anammox and denitrification.

After MIMS test, the following columns of data were obtained: *Time, ms, N28, N29, N30. Time* is the running time of MIMS (hour: min: sec); *ms* is instantaneous millisecond; *N28, N29*, and *N30* are the electrical signal values of generated $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ (unit: μ mol). The unit of denitrification and anammox rate is nmol/g/h, where g is the dry weight of sediment in the injection vial. It should be noted that the original units need to be converted (μmol $\rightarrow$ nmol; ms $\rightarrow$ h) during the calculation.

Step 3: calculation of denitrification and anammox rate

Revision of the methods to calculate the potential rates of both anammox and denitrification based on the previous studies [3,4], which mainly considered the effect of the coupling process of anammox

and DNRA on the total $^{30}N_2$ production. The revised calculation method is as follows [5]:

$$P_{29}=A_{29}+D_{29} \quad (1)$$

$$P_{30}=A_{30}+D_{30} \quad (2)$$

$$A_{29}=A_{total}\times F_1 \quad (3)$$

$$D_{29}=D_{30}\times 2(1-F_1)/F_1 \quad (4)$$

$$F_1=C_{15NO3^-}/\left(C_{14NO3^-}+C_{15NO3^-}\right) \quad (5)$$

Where P represents the electrical signal value of the product; A represents anammox rate; D represents denitrification rate; subscripts 29 and 30 indicate that the products are $^{29}N_2$ and $^{30}N_2$, respectively; A_{29} is the production rate of $^{29}N_2$ in the anammox reaction process; A_{30} is the production rate of $^{30}N_2$ in the anammox reaction process ($^{15}NH_4^+$ is mainly from the dissimilatory reduction of nitrate to ammonium (DNRA) process: $^{15}NO_3^- \rightarrow {}^{15}NH_4^+$); A_{total} is the production rate of total N_2 (include $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) in anammox reaction process; F_1 is the percentage of $^{15}NO_3^-$ concentration in the total NO_3^- concentration; C_{14NO3^-} is the concentration in the injection bottle after 48 h of pre culture (unit: μmol/L); C_{15NO3^-}=100 μmol/L. N_{29} and N_{30} are generated by the combination of ^{14}N and ^{15}N, and are random combinations of ^{14}N and ^{15}N in $^{14}NO_3^-$,$^{15}NO_3^-$,$^{14}NH_4^+$ and $^{15}NH_4^+$.

Measurement and calculation of DNRA rate

Step 1: sample pretreatment

The pretreatment steps of DNRA rate measurement were the same as those of denitrification and anammox rate measurement. The experimental group for DNRA rate measurement was set as follows: take another group (3) $^{15}NO_2^-/{}^{15}NO_3^-$ which injected with 50% saturated $ZnCl_2$ solution and purged with helium for about 5 min to remove the generated N_2, and then 200 μL of hypobromite iodine solution oxidant was injected to oxidize the $^{15}NH_4^+$ generated by DNRA process to $^{29}N_2$ and $^{30}N_2$.

Step 2: preparation of oxidant

The preparation of hypobromite iodine solution is as follows: six hundred microliters of NaOH (16 mol/L) solution was placed in a mixture of ice and water to cool it down below 5°C and then 120 mL of bromine water (Br_2) was added dropwise to the NaOH solution with the continuous stirring to keep the low temperature until the Br_2 is exhausted. After that, the mixed solution was put in the refrigerator (temperature 3–5 °C) to allow enough time (about a week) to form NaBr crystals and then precipitate completely. Finally, the resulting supernatant was mixed with equal volume of 0.2% potassium iodide (KI) solution (stabilizer) [6].

Step 3: DNRA rate calculation

The rate of DNRA was calculated according to the following equation:

$$R_{DNRA}=\frac{\left[{}^{15}NH_4^+\right]_{Final}\times Vol-\left[{}^{15}NH_4^+\right]_{Initial}\times Vol}{W\times T}$$

R_{DNRA} (nmol $^{15}N\bullet g^{-1}\bullet h^{-1}$) represents the total DNRA rate based on the ^{15}N; $[{}^{15}NH_4^+]_{Final}$ and $[{}^{15}NH_4^+]_{Initial}$ (nmol$\bullet L^{-1}$) represent the concentration of $^{15}NH_4^+$ in the slurries at the initial and the final samples, respectively; *Vol* (L) is the vial volume; *W* (g) is the weight of adding sludge; T (h) is the incubation time.

Calculation of contribution of denitrification, anammox and DNRA to TN removal and nitrate reduction

According to the calculated data, the denitrification rate S_D, anammox rate S_A and DNRA rate S_{DR} (unit: nmol/g/h) were obtained. The contributions of denitrification and anammox to TN removal were calculated by equations $S_D/(S_D+S_A)\times 100\%$ and $S_A/(S_D+S_A)\times 100\%$, respectively. Additionally, the equations for calculating the contributions of denitrification, anammox and DNRA to nitrate reduction were $S_D/(S_D+S_A+S_{DR})\times 100\%$, $S_A/(S_D+S_A+S_{DR})\times 100\%$, and $S_{DR}/(S_D+S_A+S_{DR})\times 100\%$, respectively.

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Declaration of Competing Interest

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

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