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DISSERTATION SUMMARY

Kanae Kobayashi

Title of dissertation

Coupled nitrogen and oxygen isotope effects of anaerobic ammonium oxidation (anammox)
（アナモックス細菌の窒素および酸素同位体分別の解析）

Natural abundance of stable nitrogen (N) and oxygen (O) isotopes (δ^{15}N and δ^{18}O) are invaluable biogeochemical tracers for assessing the N transformations in the environment. To fully exploit these tracers, the N and O isotope effects ($^{15}\varepsilon$ and $^{18}\varepsilon$) associated with the respective N transformation processes must be known. Anaerobic ammonium oxidation (anammox) and denitrification are the two major sinks of fixed N. In addition, anammox bacteria contribute to re-oxidation of nitrite to nitrate, because they fix CO_2 into biomass with reducing equivalents generated from oxidation of nitrite to nitrate. Nitrate production by anammox bacteria influences the nitrite and nitrate N and O isotope effects in freshwater and marine systems. Despite the significant importance of anammox bacteria in the global N cycle, $^{15}\varepsilon$ and $^{18}\varepsilon$ of anammox are not well known. Therefore, the never yet determined $^{15}\varepsilon$ and $^{18}\varepsilon$ associated with anammox were investigated in this study.

Firstly, the $^{15}\varepsilon$ were determined for '*Ca.* Scalindua sp.', '*Ca.* Jettenia caeni', and '*Ca.* Brocadia sinica' growing in highly enriched continuous enrichment cultures. Each $^{15}\varepsilon$ was calculated by the steady-state fractionation model. For the conversion of NH_4^+ to N_2, the N isotope effects ($^{15}\varepsilon_{NH4 \rightarrow N2}$) of all three species are consistent (30.9‰ to 32.7‰). This is probably because this reaction is mediated through the same enzymes such as hydrazine synthase (hzs) and hydrazine dehydrogenase (hdh) in all anammox bacteria species. On the other hand, for the conversion of NO_2^- to N_2, significant variations of the N isotope effects ($^{15}\varepsilon_{NO2 \rightarrow N2}$) were found among the three species: $^{15}\varepsilon_{NO2 \rightarrow N2} = 19.9 \pm 1.7$‰ for '*Ca.* Scalindua sp.', $^{15}\varepsilon_{NO2 \rightarrow N2} = 29.5 \pm 3.9$‰ for '*Ca.* Jettenia caeni', and $^{15}\varepsilon_{NO2 \rightarrow N2} = 5.9 \pm 4.5$‰ for '*Ca.* Brocadia sinica', respectively. This is probably because individual anammox bacteria species might possess different types of nitrite reductase. For the oxidation of NO_2^- to NO_3^-, all three anammox species exhibited pronounced inverse N isotope effects (-45.3 ± 4.2‰ to -30.1 ± 3.0‰), which agreed with the previously

reported value for '*Ca.* K. stuttgartiensis' but exceeded the values for nitrite-oxidizing bacteria (NOB).

Secondly, the $^{18}\varepsilon$ were determined for '*Ca*. Scalindua sp.', which is a putative marine species. Determination of $^{18}\varepsilon$ of anammox is more challenging because the $\delta^{18}O_{NO2}$ value is affected by abiotic O isotope exchange between NO_2^- and H_2O (k_{eq}, $^{18}\varepsilon_{eq}$) and incorporation of a water-derived O atom into NO_3^- during NO_2^- oxidation to NO_3^- ($^{18}\varepsilon_{H2O}$). In order to determine abiotic $k_{eq,}$ $^{18}\varepsilon_{eq}$, and $^{18}\varepsilon_{H2O}$, batch experiments with different $\delta^{18}O_{H2O}$ values of medium were conducted. Oxygen isotope ratio measurements of NO_2^- and NO_3^- by the azide method and denitrifier method are sensitive to the $\delta^{18}O$ of sample water. However, the influence of $\delta^{18}O_{H2O}$ on those measurements has not been quantitatively evaluated and documented so far. Therefore, the influence of $\delta^{18}O_{H2O}$ of sample on $\delta^{18}O$ analysis of NO_2^- and NO_3^- were quantitatively evaluated. We prepared NO_2^- and NO_3^- standards (with known $\delta^{18}O_{NO2-}$ and $\delta^{18}O_{NO3-}$) dissolved in waters having different $\delta^{18}O_{H2O}$ values ($\delta^{18}O_{H2O}$ = -12.6, 25.9, 56.7, and 110.1‰). The measured $\delta^{18}O$ of produced N_2O was plotted against known $\delta^{18}O_{NO2-}$ and $\delta^{18}O_{NO3-}$ values to evaluate the influence of exchange of oxygen atom with H_2O during the conversion of NO_2^- to N_2O and NO_3^- to N_2O, respectively. As a result, the degree of O isotope exchange was 10.8 ± 0.3% in the azide method and 5.5 ± 1.0 % in the denitrifier method, indicating that the azide method is more susceptible to artifacts arising from differences in the $\delta^{18}O_{H2O}$ of water than the denitrifier method. Thus, the intercept of the standard calibration curve must be corrected to account for differences in $\delta^{18}O_{H2O}$. In short, oxygen isotope ratio measurements of NO_2^- by the azide method are highly sensitive to $\delta^{18}O_{H2O}$ resulting from significant oxygen isotope exchange between NO_2^- and H_2O. Therefore, the same $\delta^{18}O_{H2O}$ as that of the sample must be used to make the NO_2^- and NO_3^- standards for the most accurate measurements. Then, the rate of abiotic O isotope exchange between NO_2^- and H_2O: k_{eq} = 1.13 × 10^{-2} (h^{-1}), as well as equilibrium isotope effects: $^{18}\varepsilon_{eq}$ = 11.9‰ were experimentally determined by conducting abiotic batch experiment with different $\delta^{18}O_{H2O}$ values of medium ($\delta^{18}O_{H2O}$ = -12.6 ~ 110.1‰). To determine $^{18}\varepsilon$ of each reaction, batch culture experiments with different $\delta^{18}O_{H2O}$ values of medium ($\delta^{18}O_{H2O}$ = -12.6 ~ 110.1‰) were conducted for '*Ca.* Scalindua sp.'. During the anammox reaction, $\delta^{18}O$ of produced NO_3^- appeared to depend on the $\delta^{18}O_{H2O}$ of medium. This observation suggested that water is oxygen source of nitrate production by anammox. Rapid increase in $\delta^{18}O$ of NO_2^- overtime in higher $\delta^{18}O_{H2O}$ medium was observed compared to abiotic exchange. This might be attributed to microbially catalyzed oxygen isotopic

exchange. A numerical model was developed for estimation of respective $^{18}\varepsilon$ of anammox reaction, and $^{18}\varepsilon$ values were determined for the first time in the world.

Finally, enzyme level $^{15}\varepsilon$ and $^{18}\varepsilon$ of nitrite oxidation and nitrate reduction was determined by using highly enriched nitrite oxidoreductase (nxr) purified from '*Ca.* Brocadia sinica'. Anammox bacteria show nitrogen inverse kinetic isotope effect during nitrite oxidation. Nitrite oxidation by anammox bacteria is owing to the enzymatic activity of nitrite oxidoreductase (nxr). Depending on its redox state nitrite oxidoreductase either oxidizes nitrite to nitrate or reduces nitrate to nitrite. However, how reversibility of nxr affects isotope effect of nitrite oxidation is not clarified yet. We tried several purification processes to purify nxr. The optimized purification process by ion chromatography, hydoroxyapatite chromatography and hydrophobic interaction chromatography carrier types of Butyl produce highly enriched nxrα and nxrβ. However, those enzymes could not be isolated in this study. Nitrite oxidation and nitrate reduction assay test were conducted with enriched nxr. During nitrite oxidation, both of δ^{15}N and δ^{18}O of nitrite decreased. In addition, δ^{15}N and δ^{18}O of produced nitrate was higher than that of nitrite. Those changes of δ^{15}N and δ^{18}O indicated that nitrite oxidation reaction itself show inverse kinetic isotope effect. As for nitrate reduction assay, the δ^{15}N and δ^{18}O increased. Thus, the reaction of nitrate reduction might be normal kinetic isotope effect. Nitrite oxidoreductase is known as reversible enzyme, but the specific activity of nitrate reduction by nxr of anammox was much smaller than that of nitrite oxidation. In conclusion, the inverse kinetic isotope effect of anammox might be attributed to reaction of nitrite oxidation itself.

These obtained dual N and O isotopic effects of anammox bacteria could provide significant insights into the contribution of anammox bacteria to the fixed N loss and nitrite reoxidation in (recycling N) in various natural environments.