（別紙様式　14）

2017 年 8 月 21 日

論文の内容の要約

氏　　名	Nguyen Cong Thuan
学位の種類	博士（農学）
学府又は研究科・専攻	連合農学研究科　　環境資源共生科学　専攻
指導を受けた大学	東京農工大学
学位論文名	Nitrification and denitrification in river ecosystems elucidated by natural abundance of stable isotopes

【論文の内容の要約】

Summary

Nitrification and denitrification in river ecosystems elucidated by natural abundance of stable isotopes

Department of Symbiotic Science of Environment and Natural Resources,
Major Chair of Environmental Conservation

NGUYEN CONG THUAN

Rivers function to transport nitrogen (N) from land to ocean accompanied with the diverse biogeochemical processes occurring such as mixing, assimilatory uptake, nitrification, heterotrophic denitrification, and N sedimentation. The question is what can be useful tools to get insights into the occurrence of N biogeochemical processes in rivers. Concentrations of N compounds can provide the fundamental information on their enrichment levels, but they cannot always provide information on the occurring N process. The rates of the N processes can be estimated in the laboratorial experiments, but the obtained results might not reflect truly their actual rates in natural conditions in rivers. The N processes taking place result in isotopic fractionations between substrates and products (expressed as isotopic fractionation factors; $^{15}\varepsilon$ and $^{18}\varepsilon$ are isotopic fractionation factors for nitrogen and oxygen, respectively), and isotopic fractionation factors can be unique and different for the different N processes. Therefore, the observation of the changes and differences in isotopic compositions for nitrogen ($\delta^{15}N$)

and oxygen ($\delta^{18}O$) among N compounds is expected to be a useful tool to understand the occurrence of the N processes in rivers. In this study I measured natural abundance of stable isotopes of N compounds to elucidate N dynamics in rivers in Japan.

The Tama River is an urban river with high nitrous oxide (N_2O) concentrations. The microbial process responsible for the high N_2O concentrations along the Tama River, however, is not fully identified due to the difficulties in elucidation of the N_2O production process. I measured the concentrations and isotopic ratios of N_2O and its substrates of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) to identify the dominant microbial process of N_2O production. I also measured the abundances of functional genes of nitrifiers (*amoA*-bacteria) and denitrifiers (*nirK*, *nirS*, *nosZ* clade I, *nosZ* clade II), dissolved organic carbon (DOC), and protein and humic components of dissolved organic matter (DOM) to assess whether these parameters can support the interpretations of N_2O production processes based on isotopic data. Surface water samples were collected at four stations (Stations 3–6) on 24 October, 2014 and all eight stations (Stations 1–8) on 13 November, 2014. Based on characteristics of EC and locations of stations, we named Stations 1–5 as downstream (DS) stations and Stations 6–8 as midstream (MS) stations. 1.5–2.0 L of surface water was filtered using a 2.7 μm pre-filter followed by the filtration with a 0.20 μm membrane filter unit. Concentrations of NH_4^+, NO_2^- and NO_3^- were analyzed using an autoanalyzer with colorimetric methods. Concentrations of DOC as non-purgeable organic carbon were analyzed using a TOC analyzer. Samples for N_2O were collected in 30 mL glass serum bottles. Concentrations of N_2O were measured using GC-ECD. Characteristics of DOM were assessed using fluorescence spectroscopy for excitation–emission matrices (EEMs). The EEMs permit identification of the fluorescence component types (i.e., humic-like and protein-like) using the peak-picking method. The EEMs were measured using a fluorospectrometer. DNA extraction from the Sterivex filter was done as described by Somerville et al. (1989) using a Fast DNA™ Spin Kit. Abundances of functional genes were quantified by real-time polymerase chain reaction (PCR) with a CFX96 Touch™ Real-Time PCR Detection System. Isotopic signatures ($\delta^{15}N$ and $\delta^{18}O$) of NO_3^- were analyzed using the denitrifier method. For the analysis of $\delta^{15}N$ of NH_4^+, NH_4^+ captured onto a glass-fiber filter by the diffusion method was oxidized to NO_3^- using persulfate reagent, and then

the NO_3^- were analyzed using the denitrifier method. The $\delta^{15}N$ and $\delta^{18}O$ of NO_2^- were analyzed using the azide method. The $\delta^{18}O$ of H_2O was analyzed using the modified azide method. The measurements of $\delta^{15}N$ and $\delta^{18}O$ were conducted with a system of Purge-and-Trap-Gas Chromatography-Isotope Ratio Mass Spectrometry (PT-GC-IRMS). The dissolved N_2O isotopic analysis was performed using an isotope ratio monitoring mass spectrometer (Toyoda et al., 2015). The microbial processes of N_2O production were identified by comparing observed $\delta^{15}N$ and $\delta^{18}O$ of N_2O with expected $\delta^{15}N$ and $\delta^{18}O$ of N_2O of different N_2O production processes of denitrification, nitrification, nitrifier-denitrification, and nitrite reduction by nitrifiers. The expected $\delta^{15}N$ and $\delta^{18}O$ of N_2O were estimated as subtracting the $^{15}\varepsilon$ and $^{18}\varepsilon$ for processes of N_2O production from $\delta^{15}N$ or $\delta^{18}O$ of substrates of NH_4^+, NO_2^-, and NO_3^-. I used the $^{15}\varepsilon$ or $^{18}\varepsilon$ for processes of N_2O production from reported values. The results showed that DS stations had the super saturation of N_2O (246.6 to 3423.3%), therefore, I only discussed N_2O production processes at DS stations. At DS stations, concentrations of NH_4^+, NO_2^-, and NO_3^- were 30.5 ± 1.8 μM (Mean ± S.E.M), 8.9 ± 2.0 μM, and 302.3 ± 27.8 μM, respectively. At DS stations, abundances of *amoA, nirK, nirS, nosZ* clade I, and *nosZ* clade II were $2.8 \times 10^5 \pm 5.9 \times 10^4$ copies/mL, $6.6 \times 10^5 \pm 2.1 \times 10^5$ copies/mL, $6.1 \times 10^5 \pm 1.1 \times 10^5$ copies/mL, $1.1 \times 10^6 \pm 2.1 \times 10^5$ copies/mL, and $3.5 \times 10^4 \pm 8.3 \times 10^3$ copies/mL, respectively. At DS stations, DOC concentration was 149.3 ± 10.1 μM. The fluorescence intensities of peaks A, C, T, and B were 20 ± 0.5 QSU mg^{-1} L^{-1}, 18.2 ± 0.7 QSU mg^{-1} L^{-1}, 11.4 ± 0.7 QSU mg^{-1} L^{-1}, 7.8 ± 0.5 QSU mg^{-1} L^{-1}, respectively. At DS stations, $\delta^{15}N$ values of NH_4^+, NO_2^-, NO_3^- and N_2O were 26.1 ± 1.2‰, 1.6 ± 1.4‰, 13.7 ± 0.5‰, and -1.8 ± 1.7‰, respectively. $\delta^{18}O$ values of NO_2^-, NO_3^-, N_2O, and H_2O were 8.2 ± 0.8‰, -1.1 ± 0.2‰, 39.7 ± 1.7‰, and -11.7 ± 0.2‰, respectively. Comparing the observed $\delta^{15}N$ and $\delta^{18}O$ of N_2O with the expected $\delta^{15}N$ and $\delta^{18}O$ of N_2O at DS stations, I found all observed $\delta^{15}N$ of N_2O data fell within the expected range of $\delta^{15}N$ for N_2O production by denitrification, while the observed $\delta^{15}N$ of N_2O data did not fall within the expected range of $\delta^{15}N$ for N_2O production by nitrification, nitrifier-denitrification, and nitrite reduction by nitrifiers. The six out of eight samples had the observed $\delta^{18}O$ of N_2O in the expected range of $\delta^{18}O$ for N_2O production by denitrification, while the observed $\delta^{18}O$ of N_2O_{Net} did not fall within the expected ranges of $\delta^{18}O$ for N_2O production by nitrification, and nitrite reduction by nitrifiers. Both $\delta^{15}N$ and $\delta^{18}O$ of

N_2O fell within the expected $\delta^{15}N$ and $\delta^{18}O$ ranges for N_2O production by denitrification, thus I concluded that denitrification was the dominant process of N_2O production along this River. A positive correlation between *nirK* gene abundance and N_2O concentration supported the importance of denitrification for the N_2O production. Since no significant correlation between N_2O concentration and DOC and fluorescence intensities of protein peaks and humic peaks, we infer that the DOC and protein and humic components of DOM did not control dissolved N_2O, but at least protein-DOM (with high lability and bioavailability) supported the occurrence of denitrification.

In rivers, dissolved inorganic nitrogen (DIN; namely NH_4^+, NO_2^-, and NO_3^-) concentrations are controlled by sources and *in situ* processes. It is difficult with the DIN concentration data only to recognize the sources and biogeochemical processes. Although the $\delta^{15}N$ and $\delta^{18}O$ of DIN species cannot be fully conservative due to the isotopic fractionations, which would compromise their ability of the source apportionments, their non-conservative behaviors can provide insight into the predominant, *in-situ* processes influencing DIN dynamics in rivers with the help of isotopic fractionation factors for the target N process. Despite of the importance of isotopic fractionation factors on DIN in the river, the reports of the isotopic fractionation factors are scarce. The spiraling metrics of N biogeochemistry such as uptake length (S_w, m), uptake velocity (V_f, m/s), and areal uptake rates (U, μmol/m^2 min) of nutrient nitrogen atoms can be obtained by the Lagrangian approach and the spiraling metrics can provide information on the levels of DIN production and consumption. The levels of DIN production and consumption determining the spiraling metric must also influence concentrations and isotopic signatures of DIN species and then isotopic fractionations. Therefore, we hypothesized that isotopic fractionations are related to the spiraling metrics for DIN species. If this is true, the isotopic fractionation factors, and further, isotopic signatures of DIN can be used as a parameter of the

spiraling metrics. To test this hypothesis, I estimated isotopic fractionation factors for net NH_4^+ consumption ($^{15}\varepsilon_{AC}$) and spiraling metrics of uptake length, uptake velocity, and areal uptake rates for NH_4^+ in the Fuji River. I also elucidated biological processes causing changes in DIN concentrations with distances based on concentrations and isotope ratios of DIN species. Water samples were collected on June 25, October 15, November 12, and December 7, 2015. In each sampling period, 17–21 sampling points were selected. Distance between two next sampling points was about 300 m (210 to 466 m). The N fluxes were monitored in water mass by the longitudinal Lagrangian sampling approach. Concentrations and isotope ratios of DIN species (NH_4^+, NO_2^-, and NO_3^-) were measured in the water samples. The $^{15}\varepsilon_{AC}$ was estimated as the slope for the linear regression between natural logarithm of NH_4^+ concentration versus $\delta^{15}N$ of NH_4^+ using the Rayleigh model. The spiraling metrics (S_w, V_f, and U) for NH_4^+ were estimated after Hensley et al. (2014). The results showed that concentrations of NH_4^+ were 9.4 ± 2.2 µM in June, 9.0 ± 0.4 in October, 14.6 ± 2.0 µM in November, and 22.9 ± 2.1 µM in December. Concentrations of NO_2^- were 1.8 ± 0.1 µM in June, 2.0 ± 0.0 µM in October, 3.1 ± 0.1 µM in November, and 3.0 ± 0.2 µM in December. Concentrations of NO_3^- were 80.9 µM to 106.8 µM, with 85.3 ± 2.4 µM in June, 93.0 ± 0.6 µM in October, 104.0 ± 1.5 µM in November, and 104.9 ± 1.7 µM in December. Values of S_w, V_f and U for NH_4^+ were 7.9 to 29.3 km, 10.5 to 56.7 cm/h, and 2.4 to 4.9 mmol/m^2/h, respectively. The $\delta^{15}N$ of NH_4^+ was 24.6 ± 2.3‰ in June, 22.7 ± 0.3‰ in October, 24.3 ± 1.0‰ in November, and 21.4 ± 1.2‰ in December. In December, $\delta^{15}N$ of NO_2^- was -9.0 ± 0.4‰ and $\delta^{18}O$ of NO_2^- was 6.1 ± 0.2‰. $\delta^{15}N$ of NO_3^- were 8.1 ± 0.2‰ in June, 7.9 ± 0.0‰ in October, 8.3 ± 0.1‰ in November, and 7.4 ± 0.1‰ in December. $\delta^{18}O$-NO_3^- were -1.2 ± 0.3‰ in June, -1.2 ± 0.0‰ in October, -1.6 ± 0.3‰ in

November, and -1.6 ± 0.2‰ in December. The $^{15}\varepsilon_{AC}$ values were estimated 8.9 ± 1.2‰, 6.2 ± 1.7‰, 5.3 ± 2.0‰, and 4.0 ± 1.4‰ for June, October, November, and December respectively. Concentrations of NH_4^+ decreased gradually with distances in all sampling times, whereas concentrations of NO_3^- increased gradually with distances. The decrease in NH_4^+ concentrations accompanied with the increase in $\delta^{15}N$ of NH_4^+ in all sampling times, indicating NH_4^+ was consumed by biological processes. The significant positive correlations between $\delta^{15}N$ of NH_4^+ and $\delta^{15}N$ of NO_3^-, and the lower $\delta^{15}N$ of NO_2^- than those of NH_4^+ and NO_3^-, illustrated that nitrification was the main process for NH_4^+ consumption and NO_3^- accumulation. Interestingly, the $^{15}\varepsilon_{AC}$ had significant positive relations with uptake velocity and rate for NH_4^+, but need more evidences to confirm the possibility that the $^{15}\varepsilon_{AC}$ can be a good parameter for spiraling metrics.

The $^{15}\varepsilon_{AC}$ was only estimated in the Fuji River and the measured $^{15}\varepsilon_{AC}$ might not be so typical one for the general river system. To confirm the possible reasons for the variations of the $^{15}\varepsilon_{AC}$, it is necessary to carry out the estimation of the $^{15}\varepsilon_{AC}$ in other rivers with different NH_4^+ concentrations. To investigate the variation of the $^{15}\varepsilon_{AC}$ across rivers with different NH_4^+ concentrations, the investigation in four other large rivers (the Saigawa River, the Arakawa River, the Chikuma River, and the Tama River) was conducted in 2016. The methods of sampling and measurements in these four rivers were as same as the methods was done in the Fuji River. In rivers surveyed in 2016, only the Tama River had the gradual decrease in NH_4^+ concentrations with distances, therefore, the $^{15}\varepsilon_{AC}$ and spiraling metrics for NH_4^+ were only estimated in the Tama River. The S_w, V_f, and U for NH_4^+ were estimated 2.4 km, 159.2 cm/h, and 8.8 mmol/m^2h, respectively in the Tama River. The $^{15}\varepsilon_{AC}$ was estimated as 9.0 ± 0.7‰ in the Tama River. Across all surveyed rivers, the variations of $\delta^{15}N$ and/or $\delta^{18}O$ of NH_4^+, NO_2^-, and NO_3^- were large (-9.0‰ to 38.7‰ for $\delta^{15}N$, -17.6‰ to 6.1‰ for $\delta^{15}N$ of NO_2^-, 1.6 to 15.3‰ for $\delta^{15}N$ of NO_3^-, 4.0‰ to 12.1‰ for $\delta^{18}O$ of NO_2^-, and -3.5‰ to 1.0‰ for $\delta^{18}O$ of NO_3^-), and the significant positive correlations between $\delta^{15}N$ of NH_4^+, NO_2^-, and NO_3^- with their concentrations were found. Data in all surveyed rivers showed that

in rivers (the Saigawa River, the Arakawa River, and the Chikuma River) with low NH_4^+ concentrations (ca. 1.5 μM), NH_4^+ consumption was considered to be negligible which was confirmed by the constant NH_4^+, NO_2^- concentrations with distances. In rivers (the Fuji River and the Tama River) with moderate-to-high NH_4^+ concentrations (above 5 μM), nitrification was the dominant process for NH_4^+ consumption and NO_3^- production. The decrease in $\delta^{15}N$ in the order of NH_4^+, NO_3^-, and NO_2^- was an indicator for the dominant nitrification in large rivers. No clear evidence from concentration and isotope data of DIN species for occurrence of assimilation and denitrification were found in large rivers. The long area uptake length for NH_4^+ (2.4 km to 29.2 km in rivers with moderate-to-high NH_4^+ concentrations) together with insignificant NO_3^- consumption implied nutrient N removal efficiency was low in the large rivers. In large rivers, production and consumption rates are not high, resulting in the quite small changes in concentrations and isotope ratios of N compounds. For example, the largest changes in the 5 -km distance were smaller than 6 μM and 6‰ for concentrations and isotope ratios of NH_4^+, respectively (along the Fuji River) across all rivers surveyed. Combining data in Fuji River and Tama River, the significant negative correlation between $^{15}\varepsilon_{AC}$ and S_w was found, confirming that the spiraling metrics for NH_4^+ controlled significantly the $^{15}\varepsilon_{AC}$. It is further suggested that the estimated $^{15}\varepsilon_{AC}$ can predict NH_4^+ uptake levels in rivers.

This study demonstrates that the isotopic measurements of N compounds are the powerful tools to get insights into the occurrence of the N biogeochemical processes in rivers. The isotope ratios of N_2O and its substrates of NH_4^+, NO_2^-, and NO_3^- evidenced that denitrification was the dominant process of N_2O production along the Tama River. I did the first study of estimation of the $^{15}\varepsilon$ for ammonia consumption with values of 4.0 to 9.0‰ in the river environments. I found firstly that the $^{15}\varepsilon$ for net NH_4^+ consumption had the significant relations with uptake length, uptake velocity, and area uptake rate for NH_4^+. Thus, it is possible to consider that the $^{15}\varepsilon$ for N consumption can be a good proxy to predict levels of N removal in rivers.