Journal of Visualized Experiments

Measurement of the potential rates of dissimilatory nitrate reduction to ammonium based on 14NH4+/15NH4+ analyses via sequential conversion to N2O

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59562R1
Full Title:	Measurement of the potential rates of dissimilatory nitrate reduction to ammonium based on 14NH4+/15NH4+ analyses via sequential conversion to N2O
Keywords:	Nitrogen cycle; Dissimilatory nitrate reduction to ammonium (DNRA); 15N tracer; Diffusion method; Persulfate oxidation; quadrupole GC/MS; Salt marsh
Corresponding Author:	Megumi Kuroiwa Chuo Daigaku Tokyo, JAPAN
Corresponding Author's Institution:	Chuo Daigaku
Corresponding Author E-Mail:	y-suwa@bio.chuo-u.ac.jp
Order of Authors:	Megumi Kuroiwa
	Keitaro Fukushima
	Kazuma Hashimoto
	Yukiko Senga
	Tsubasa Sato
	Chie Katsuyama
	Yuichi Suwa
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tokyo, Japan

Dec 14, 2018

Dear Dr. Myers,

We submit the manuscript herewith entitled “Measurement of the potential rates of dissimilatory nitrate reduction to ammonium (DNRA) based on $^{14}NH_4^+/^{15}NH_4^+$ analysis by sequential conversion and detection with quadrupole GC/MS”.

1) All authors have read the submitted version of the manuscript and agree to submit the work to JoVE.

2) This work has not been published before and is not being considered for publication by another journal.

3) In this paper, we provide the detailed procedure for the measurement of potential DNRA rate and applied it to the salt marsh sediments. Briefly, the potential DNRA rate can be calculated from the ^{15}N-labeled ammonium ($^{15}NH_4^+$) accumulation rate in ^{15}N-labeled nitrate ($^{15}NO_3^-$) addition incubation. Determination of non-labeled and labeled ammonium concentrations is comprised of several sequential steps, including the collection and conversion of the ammonium. These steps are basically based on previous studies, we modified several procedures. Firstly, in the step of collecting NH_4^+, we shortened the incubation period and lowered temperature. Secondly, we made a few modifications to the method for conversion about preservation and preparation of the bacterial cells required in this procedure. These modifications allow to shorten the required time for a series of the experiments. In this method, NH_4^+ is finally converted to N_2O and it enable to determine concentration and isotope ratio of NH_4^+ by quadrupole GC/MS since N_2O has low atmospheric background. GC/MS is less expensive and easy to manage compared with IRMS, which has been typically used in $^{15}NH_4^+$ studies. The representative result demonstrated the proposal procedures effective to determine the NH_4^+ isotope ratio and concentration especially for samples with relatively high concentration of ammonium. Because of these, we believe that proposed method will gain broad attention from the scientific community and make a contribution to JoVE.

We appreciate if the manuscript could be reviewed and considered for publication.

Kind regards,

Megumi Kuroiwa, Ph.D.
Assistant Professor, Department of Biological Sciences, Chuo University
1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan.
Phone: +81-3-3817-7185
E-mail: mkuro.25w@g.chuo-u.ac.jp

TITLE:

Measurement of the Potential Rates of Dissimilatory Nitrate Reduction to Ammonium Based on $^{14}NH_4^+$/$^{15}NH_4^+$ Analyses via Sequential Conversion to N_2O

AUTHORS & AFFILIATIONS:

Megumi Kuroiwa[1], Keitaro Fukushima[2,3], Kazuma Hashimoto[2], Yukiko Senga[4], Tsubasa Sato[4], Chie Katsuyama[5], Yuichi Suwa[1]

[1]Department of Biological Sciences, Faculty of Science and Engineering, Chuo University, Tokyo, Japan

[2]Faculty & Graduate School of Urban Environmental Sciences, Tokyo Metropolitan University, Tokyo, Japan

[3]Center for Ecological Research, Kyoto University, Kyoto, Japan

[4]Department of Chemistry, Faculty of Science, Toho University, Chiba, Japan

[5]Graduate School of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan

Corresponding Author:

Megumi Kuroiwa (mkuro.25w@g.chuo-u.ac.jp)

Tel: +81-3-3817-7185

Email Addresses of Co-authors:

Keitaro Fukushima (ktaro.f@gmail.com)

Kazuma Hashimoto (tgs.bloom@gmail.com)

Yukiko Senga (yukiko.senga@sci.toho-u.ac.jp)

Tsubasa Sato (t.casiare@gmail.com)

Chie Katsuyama (ckatsu@hiroshima-u.ac.jp)

Yuichi Suwa (y-suwa@bio.chuo-u.ac.jp)

KEYWORDS:

nitrogen cycle, dissimilatory nitrate reduction to ammonium (DNRA), ^{15}N tracer, diffusion method, persulfate oxidation, quadrupole GC/MS, salt marsh

SUMMARY:

A series of methods to determine the potential DNRA rate based on $^{14}NH_4^+$/$^{15}NH_4^+$ analyses is provided in detail. NH_4^+ is converted into N_2O via several steps and analyzed using quadrupole gas chromatography–mass spectrometry.

ABSTRACT:

The importance of understanding the fate of nitrate (NO_3^-), which is the dominant N species transferred from terrestrial to aquatic ecosystems, has been increasing because global nitrogen loads have dramatically increased following industrialization. Dissimilatory nitrate reduction to ammonium (DNRA) and denitrification are both microbial processes that use NO_3^- for respiration. Compared to denitrification, quantitative determinations of the DNRA activity have been carried out only to a limited extent. This has led to an insufficient understanding of the importance of DNRA in NO_3^- transformations and the regulating factors of this process. The objective of this

paper is to provide a detailed procedure for the measurement of the potential DNRA rate in environmental samples. In brief, the potential DNRA rate can be calculated from the ^{15}N-labeled ammonium ($^{15}NH_4^+$) accumulation rate in $^{15}NO_3^-$ added incubation. The determination of the $^{14}NH_4^+$ and $^{15}NH_4^+$ concentrations described in this paper is comprised of the following steps. First, the NH_4^+ in the sample is extracted and trapped on an acidified glass filter as ammonium salt. Second, the trapped ammonium is eluted and oxidized to NO_3^- via persulfate oxidation. Third, the NO_3^- is converted to N_2O via an N_2O reductase deficient denitrifier. Finally, the converted N_2O is analyzed using a previously developed quadrupole gas chromatography–mass spectrometry system. We applied this method to salt marsh sediments and calculated their potential DNRA rates, demonstrating that the proposed procedures allow a simple and more rapid determination compared to previously described methods.

INTRODUCTION:

The artificial synthesis of nitrogen fertilizer and its widespread application have greatly perturbed the global nitrogen cycle. It is estimated that the transfer of reactive nitrogen from terrestrial to coastal systems has doubled since pre-industrial times[1]. A significant portion of fertilizers applied to a given field is washed away from the soil to rivers or groundwater, primarily as NO_3^- [2]. This may cause environmental problems such as drinking water pollution, eutrophication, and the formation of hypoxia. NO_3^- in water environments is removed from or retained in the ecosystem via biological assimilation and various microbial dissimilatory processes. Denitrification and anammox are known to be major microbial removal processes for NO_3^-. Denitrification is the microbial reduction of NO_3^- to gaseous N products (NO, N_2O, and N_2) coupled with the oxidation of an electron donor, such as organic substances, thereby reducing the risk of the above-mentioned problems. Anammox also produces N_2 from NO_2^- and NH_4^+; therefore, it removes inorganic N from an ecosystem. Conversely, DNRA works to retain N in an ecosystem; it is generally accepted that DNRA is performed primarily by fermentative bacteria or chemolithoautotrophic bacteria and that they reduce dissimilatory NO_3^- to bioavailable and less mobile NH_4^+.

Studies on DNRA have primarily been performed in marine or estuarine ecosystems, such as oceanic or estuarine sediments and water, salt or brackish marsh soil, and mangrove soil. Coastal or marine ecosystems are important as reservoirs for removing NO_3^- from terrestrial ecosystems, and in previous studies DNRA has been shown to contribute over a very wide range of NO_3^- removal (0–99%)[3-18]. Further, the existence of DNRA has been demonstrated in a wide range of environments including freshwater environments[19], rice paddy soils[20], and forest soils[21]. While these studies have shown that DNRA is potentially comparable to denitrification for NO_3^- removal, studies measuring the DNRA activity are still very limited compared to those measuring denitrification.

The DNRA rate has been evaluated using ^{15}N-labeling techniques in conjunction with data analysis via analytical or numerical models. One analytical solution to calculate the DNRA rate is based on the increase in the ^{15}N enrichment of the NH_4^+ pool after the addition of $^{15}NO_3^-$ as a tracer. ^{15}N-labeled NO_3^- is added to a sample and incubated, and the DNRA rate can then be calculated from the concentration and isotope ratio changes in NH_4^+ before and after a certain

period of time. In this paper, a method to quantify the NH_4^+ concentration and the isotope ratio, which are required to calculate the DNRA rate, is described in detail. Basically, the method reported here is a combination of several previously reported techniques[22-26] with modifications added to some procedures. The method is comprised of a series of five component procedures: (1) incubation of an environmental sample with the amendment of a stable isotope tracer, $^{15}NO_3^-$, (2) extraction and recovery of NH_4^+ using a "diffusion procedure" with modifications, (3) persulfate oxidation of NH_4^+ in the sample, consisting of indigenous NH_4^+ and $^{15}NH_4^+$ derived from $^{15}NO_3^-$ via DNRA activity, into NO_3^- and $^{15}NO_3^-$, (4) subsequent microbial transformation of NO_3^- and $^{15}NO_3^-$ to N_2O isotopomers via the modified denitrifier method, and (5) quantification of the N_2O isotopomers using gas chromatography–mass spectrometry (GC/MS). In the following section, first, the preparation for procedures (2) and (4) is described and then, subsequently, all five component procedures are described in detail.

PROTOCOL:

1. Preparation of a PTFE envelope for quantitatively capturing gaseous NH_3

1.1. Place a 60-mm piece of polytetrafluoroethylene (PTFE) tape (25 mm in width) on a small sheet of aluminum foil (approximately 300 mm x 450 mm in size, wiped with ethanol).

1.2. Ash a glass fiber filter (10 mm in diameter with a pore size of 2.7 µm) at 450 °C for 4 h in a muffle furnace. Place the glass fiber filter a little above the midpoint of the longer axis of the tape (**Figure 1a**).

1.3. Spot 20 µL of 0.9-mol/L H_2SO_4 on the center of a GF/D filter, and immediately fold the PTFE tape using two tweezers: flat-ended stamp tweezers and straight-ended tweezers. The following steps, steps 1.4–1.7, are shown in **Figure 1** and should be conducted swiftly.

1.4. Flip the PTFE tape over the GF/D filter at the dotted line shown in **Figure 1a** to form the shape shown in **Figure 1b**.

1.5. Seal both sides by folding and then tightly pressing the edge with the tweezers (**Figure 1c**). Do not press too hard, and do not scratch the PTFE tape.

1.6. Fold the open end with the tweezers, and then press the edge with the tweezers (**Figure 1d**).

1.7. Seal the open end by tightly pressing the edge with the tweezers (**Figure 1e**). The GF/D filter should not be pressed during this procedure.

2. Preparing the biomass of a nitrous oxide reductase deficient denitrifier, *Pseudomonas chlororaphis* subsp. *aureofaciens* ATCC13985, for the denitrifier method

2.1. Streak a 20% glycerol stock of *Pseudomonas chlororaphis* subsp. *aureofaciens* ATCC13985 on 1/4 strength tryptone soy broth (TSB) agar plates. Incubate the plates at 25 °C for 2–3 days.

2.2. Transfer a singles colony of *P. chlororaphis* to a small test tube containing 5 mL of autoclaved TSB medium and culture aerobically (without shaking) for a day at 25 °C in dark until obtaining maximum growth; this will be used as the preculture.

2.3. Transfer 3 mL of the preculture to a 1-L bottle with a silicon rubber stopper containing 1 L of freshly prepared autoclaved modified TSB supplemented with 10 mmol/L KNO_3[23]. Incubate the bottle while agitating using a stirrer under dark conditions at 25 °C. After cultivating for 8 h, replace the silicon rubber stopper with a screw cap and close tightly. Continue the cultivation in anoxia overnight.

2.4. Centrifuge the culture at 18,800 x *g* for 15 min at 4 °C to obtain biomass pellets.

2.5. Wash the packed biomass three times with 30 mL of Dulbecco's phosphate-buffered saline (D-PBS(-), pH 7.5), to completely eliminate the NO_3^-. The conditions of the centrifugation are same as in Step 2.3.

2.6. After washing, re-suspend the packed biomass with 30 mL of D-PBS(-). Use 1 mL of the suspension to determine the cell density by measuring OD_{600}. Pipette 1 mL aliquots of the remaining suspension into sterile cryovials containing 0.8 mL of 45% glycerol. Preserve the prepared glycerol stocks at −80 °C until use (see section 6).

3. Elimination of oxygen, nitrite, and nitrate from the sample sediment

3.1. Weigh 3.0 g (wet weight) of sediment into a 20 mL glass vial and add 9.0 mL of surface water to suspend it (25% w/w slurry).

3.2. Seal the vial with a black butyl rubber stopper (washed with ion-exchanged water and sterilized via autoclaving) and an aluminum cap.

3.3. Purge the suspension and replace the headspace air with Ar (>99.99%) at 0.6 L/min for 20 min using a manifold.

3.4. Replace the Ar headspace gas with ultra-pure (>99.99995%) He by vacuuming for 90 s and charging He for 30 s. Repeat this procedure four times. Set the headspace gas pressure to 1.5 atm.

3.5. Incubate the vials at 20 °C overnight with shaking at 150 rpm under dark conditions using a constant temperature shaker to eliminate the remaining oxygen, nitrate, and nitrite in the sediment suspension and headspace gas.

4. Time course experiment for determining DNRA rate

4.1. Replace the headspace gas with fresh ultra-pure He using the same procedure as in step 3.5 but without the four repetitions.

4.2. Add labeled and non-labeled substrates to each vial according to **Table 1** through the butyl rubber stopper using a gastight syringe. Purge the previously prepared substrate solutions in adequately sized glass vials using ultra-pure He with the pressure of the headspace set to 1.5 atm to avoid air contamination. Avoid the unintentional injection of any amount of air during the injection procedures.

4.3. Incubate vials at 20 °C with shaking at 150 rpm. Add the substrates to each vial according to **Table 1** and incubate for 1 h, 3 h, and 5 h. After incubation is halted, subject the sediment suspension in the vials to the following procedures: steps 4.4–4.9.

4.4. Remove the aluminum cap and butyl rubber stopper from each vial. Then, add KCl (ashed at 450 °C for 4 h) to the sediment suspension up to a final concentration of approximately 2 mol/L to ensure the recovery of NH_4^+ from the sediment. Close the vials with a butyl rubber stopper and an aluminum closure.

4.5. Shake the sediment at 150 rpm for 1 h at 4 °C under dark conditions to extract the NH_4^+.

4.6. Transfer the entire sediment suspension in the vial to a 50 mL plastic centrifuge tube, and centrifuge at 10,000 x *g* for 10 min at 4 °C.

4.7. Rinse the internal wall of a freshly opened 10-mL disposable syringe, and attach it to a freshly opened disposable cellulose acetate membrane filter (pore size 0.22 µm, 25 mm in diameter). Then, rinse the membrane filter with 1 mL of supernatant. Place the rinsed syringe–filter unit onto a 20-mL wide-mouth polypropylene (PP) bottle.

4.8. Filter the remaining supernatant through an acetate membrane filter into the 20-mL PP bottle. Store the extracts extracts at −20 °C until further analysis.

5. Capturing diffused NH_4^+ in 2M H_2SO_4 absorbed to the GF/D filter in the PTFE envelope and the persulfate oxidation of NH_4^+ to NO_3^-

5.1. Prepare standard solutions of $^{14}NH_4Cl$ with concentration gradients of 0 µmol/L, 10 µmol/L, 40 µmol/L, 100 µmol/L, 200 µmol/L, 400 µmol/L, and 500 µmol/L. For the ^{15}N ratio analysis, fix the total concentration of NH_4^+ to 200 µmol/L and prepare isotope ratios of 100:0, 99.5:0.5, 99:1, 93:7, 90:10, 50:50, and 10:90 with pure $^{14}NH_4Cl$ and $^{15}NH_4Cl$ standard solutions.

5.2. Transfer 30 mg of MgO (ashed at 450 °C for 4 h) to a 20-mL glass vial, and place the PTFE envelope in the vial.

5.3. Transfer 5 mL of a sample or standard into the vial containing the MgO and the PTFE envelope, and immediately close with a gray butyl rubber stopper. Seal with an aluminum cap. If the concentration of NH_4^+ is expected to exceed 500 µmol/L, dilute the sample to below 500 µmol/L.

5.4. Shake the vials at 150 rpm for 3 h at 4 °C under dark conditions.

5.5. Remove the aluminum cap and the butyl rubber stopper. Take the PTFE envelope out of the vial using point-ended tweezers, thoroughly rinse the envelope and the tweezers with ion-exchanged water, wipe them with a wiping paper, and then place the envelope on a fresh wiping paper.

5.6. Open the PTFE envelope with a couple of tweezers (use of both the flat-ended and point-ended tweezers is recommended) in the exact reverse order of the folding performed in steps 1.4–1.7.

5.7. Hold the peripheral area of the GF/D filter, where the H_2SO_4 is supposed to be unabsorbed, with the flat-ended tweezers, and transfer it into an 11-mL screw cap test tube with a PTFE -lined cap. Rinse the tweezers with ion-exchanged water, and wipe them with wiping paper.

5.8. Repeat steps 5.5–5.7 for the remaining envelopes.

5.9. Add 1 mL of ion-exchanged water to each of the test tubes, close the screw cap, and maintain it without shaking for at least 30 min at room temperature to completely elute the NH_4^+ from the GF/D filter. During this step, carry out the following step (step 5.10) in parallel.

5.10. Prepare the persulfate-oxidizing solution (POR) reagent[25, 26].

5.10.1. Because it cannot be stored, prepare the exact amount of POR needed for treating a single day of samples.

5.10.2. To prepare POR to treat 50 samples, pour 100 mL of ion-exchanged water into a 200-mL screw cap bottle and add 1.52 g of NaOH (nitrogen compound analysis grade), 3 g of boric acid, and 5 g of $K_2S_2O_8$ (nitrogen and phosphorus analysis grade) in that order. Immediately after adding each reagent, shake the solution until it is completely dissolved.

5.10.3. If necessary, soak the bottle in warm water to help the dissolution of the chemicals; however, attention should be paid with respect to the contamination of NO_3^- because tap water normally contains NO_3^-.

5.11. After Step 5.8, open the screw cap, add 2 mL of the POR reagent to the test tube, and close the tube tightly with a screw cap to prevent any loss or contamination during the following steps.

5.12. Stand the test tubes on a rack, wrap them in double-layered aluminum foil, and autoclave them for 1 h at 121 °C. Keep the tubes in an upright position during this step and avoid rapid changes in temperature after finishing the autoclaving.

6. Determining the NO_3^- converted from NH_4^+ by the denitrifier method using quadrupole GC/MS

6.1. Mix 100 mL of sterile 40-mmol/L phosphate buffer (pH 7.2) and 100 mL of sterile 30-mmol/L glucose aseptically (20-mmol/L phosphate and 15-mmol/L glucose; final).

6.2. Add a 1/7.2 volume of glycerol stock of *P. chlororaphis* to 200 mL of the phosphate-buffered glucose solution in a 300-mL Erlenmeyer flask, and purge with an ultra-pure He (>99.995%) stream for 1 h.

6.3. Dispense 2.0 mL of the denitrifier suspension into each 5-mL vial. Cap the vials with a gray butyl rubber stopper and an aluminum closure.

6.4. Replace the headspace air with ultra-pure He by vacuuming for 3 min and charging the He for 1 min. Set the headspace gas positive pressure to 1.3 atm to avoid unintentional air contamination.

6.5. Inject 1 mL of a sample or standard through the butyl rubber stopper using a 1-mL disposable syringe. Note the exact amount of the sample actually injected.

6.6. Incubate the vials overnight at 25 °C under dark conditions.

6.7. Inject 0.3 mL of 6-mol/LL NaOH to stop the denitrification and absorb the headspace CO_2, which will otherwise seriously disturb the N_2O analysis by GC/MS because CO_2 and N_2O have the same molecular weight.

6.8. Determine the amounts of $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ in the headspace gas using quadrupole GC/MS with a modified injection port[25]. The operating conditions used for the GC/MS analysis are shown in **Table 2**.

7. Data analysis

7.1. Derive the calibration curve for the NH_4^+ concentration from the linear relationship between the known concentration of $^{14}NH_4^+$ and the measured signal intensities of the total produced $^{44}N_2O$ + $^{45}N_2O$ + $^{46}N_2O$. Derive the calibration curve for the ^{15}N content from the linear relationship between the known atom% (*i.e.*, $^{15}N/^{14}N+^{15}N$) and the calculated atom% using a previously provided equation[27]. Calculate the concentration of $^{15}NH_4^+$ by multiplying the total NH_4^+ by the ^{15}N ratio of NH_4^+.

7.2. Calculate the potential DNRA rate using equations provided elsewhere[28-30].

REPRESENTATIVE RESULTS:

The representative results presented in this paper were derived from ^{15}N-tracing experiments of salt marsh sediments. The sampled salt marsh was newly created in the aftermath of the 2011

Great East Japan Earthquake in the Moune area of Kesen-numa city in Miyagi Prefecture, Japan. In September 2017, surface sediments (0–3 cm) were collected at two sites in the subtidal and intertidal zones. First, immediately after collection, the sediment was sieved through a 4-mm mesh to remove plant roots, shells debris, and rubble and then homogenized. The samples were stored at 4 °C until the DNRA analysis was conducted.

The incubation procedures for the $^{15}NO_3^-$ and the simultaneous determination of the $^{14}NH_4^+$ and $^{15}NH_4^+$ concentrations were carried out as described in the protocol section. An increase in the $^{15}NH_4^+$ concentration throughout the incubation period was observed for all sediments (**Figure 2**). We calculated the DNRA rates by dividing the accumulation rate of $^{15}NH_4^+$ by the isotope ratio of the NO_3^- pool[29]. The calculated rates were within the range of 24.8–177 nmol-N g^{-1} dry soil h^{-1} (**Table 3**) and were comparable to values found in previous studies. This range of obtained rates is higher than the reported values derived from similar environments including those from intertidal sediments[17], salt marshes[5, 16], and other estuarine environments[18, 33, 34], as well as from eutrophic environments such as a shallow river estuary in North Carolina[31] and the Shanghai urban river networks[32]. Conversely, Fernandes et al.[13] reported higher potential DNRA rates in organically rich mangrove soils in India. In general, DNRA is thought to be favored by a high ratio of available C to electron acceptors[35-37]. The samples demonstrating the representative results were taken from a salt marsh newly created by an earthquake, which had originally been used as a cultivation field. This particular characteristic of the samples may contribute to the observed high DNRA rate. Consistent with this speculation, the DNRA rate in the intertidal zone, which is rich in organic compounds (data not shown) compared to the subtidal zone, was higher than that in the subtidal zone (**Figure 2**, **Table 3**).

FIGURE & TABLE LEGENDS:

Figure 1: Preparation of a PTFE envelope for capturing gaseous NH_3. The PTFE envelope used in the diffusion procedure is prepared by folding PTFE tape following the instructions shown in panels **(A)–(E)**. The acidified filter inside the envelope captures the gaseous NH_3. These steps should be conducted quickly. Detailed information is given in steps 1.2–1.7 in the protocol section.

Figure 2: Change in the $^{15}NH_4^+$ concentration via the anaerobic incubation of sediments. The ^{15}N tracer incubations of the sediment samples were conducted in duplicate. The concentration of ^{15}N-NH_4^+ is shown in nmol per dry weight of sediment.

Figure 3: An example of calibration curve of low concentration N_2O. Peak area of N_2O was obtained by sum of the peak area of m/z 44, m/z 45, and m/z 46. Configurations for GC/MS analysis is shown in **Table 2.**

Table 1: Combinations of substrates amended to vials retaining approximately 11 mL of the sediment suspension. Samples were prepared in duplicate and subjected to further analyses after 1 h, 3 h, and 5 h of incubation.

Table 2: Conditions for the GC/MS analysis.

Table 3: Potential DNRA rates of the tested intertidal and subtidal sediments.

DISCUSSION:

The concentration and isotope ratio of NH_4^+ for the DNRA analysis was quantified using several methods. The concentrations and isotope ratios of NH_4^+ are generally measured separately. The NH_4^+ concentration is typically measured using colorimetric methods including an autoanalyzer[4,10,15-17]. The isotope ratio measurement has wide variations depending on its method of NH_4^+ conversion, trapping, and instrumentation for the analysis. Typical methods include the following:

(1) The NH_4^+ in the sample is converted to NH_3 via the addition of MgO or NaOH. After moving from the liquid phase to the gas phase, the NH_3 is trapped on an acidified glass filter or in an acid solution. After drying, the filter is combusted and analyzed as N_2 using an elemental analyzer/isotope ratio mass spectrometer (EA-IRMS)[11,18,22,38]. Alternatively, the captured NH_4^+ in an acid solution is collected on an adsorbent (e.g., zeolite) and is combusted and analyzed via EA-IRMS[10,12].

(2) NH_4^+ is oxidized to N_2 via hypobromite oxidation. The isotopic composition of the evolved N_2 is measured via IRMS[4,14,39] or membrane-introduction mass spectrometry (MIMS)[16,17].

(3) The NH_4^+ concentration is determined using high-performance liquid chromatography without any conversion. Because the equilibrium of NH_4^+ and NH_3 is slightly different for $^{15}NH_4^+$ and $^{14}NH_4^+$ near the pH of pK_a, the $^{14}NH_4^+$ and $^{15}NH_4^+$ concentrations can be determined based on a small shift in the retention time[6,19,40].

The method described in this paper is basically the same as approach (1) listed above, i.e., the step recovering NH_4^+ as NH_3 on a glass filter under alkaline conditions; however, it is different with respect to the following sequential NH_4^+ conversion steps. These conversion steps are based on previous studies with added modifications to shorten the required time for a series of experiments. First, we made a few modifications to the original denitrifier method. After the biomass of *P. chlororaphis* is prepared as previously described[23,24], we grow bacteria and preserve the concentrated cell suspension as a glycerol stock. This dense cell suspension can be directly used for the isotope analysis by mixing it with a buffer solution because the denitrifying activity has already been sufficiently induced. Even though further investigation is required, this modification may improve the reproducibility of the analysis because the presented method enables the mass-cultivation of denitrifier cells, which are directly available for analyses. We also modified the composition of the solution for suspending the bacterial cells, from a medium based on TSB to a phosphate-buffered glucose solution, to exclude the unnecessary components such as peptone in the original medium. This modification may reduce contamination by blank N because the phosphate-buffered glucose solution does not contain N, unlike the original medium used in previous studies; this should be tested via further analyses. In the step collecting NH_4^+ via the diffusion method, we shortened the incubation period and lowered the temperature to minimize the breakdown of organic N and any unfavorable conversion or loss of NH_4^+. The validity

of this modification was checked using the linearity of the calibration curve. We also checked that the modified temperature and incubation period did not affect the recovery of NH_4^+ (data not shown).

Another advantage of this method is that NH_4^+ is ultimately converted to N_2O, which has a low atmospheric background and can be measured using quadrupole GC/MS, which is less expensive and easier to manage than IRMS. Under the condition shown in table 2, CO_2 (m/z 44) and N_2O (m/z 44) are completely separated by GC; the retention time of these gases are 1.15 and 1.07 min, respectively. Since atmospheric concentration of N_2O is on the order of ppb, N_2O can be measured with negligible air interference even in low concentrations. The calibration curve of N_2O passes almost through the origin, demonstrating the influence on the concentration of N_2O due to atmospheric contamination is very limited (**Figure 3**). This method also has the advantage that it can quantify the $^{14}NH_4^+$ and $^{15}NH_4^+$ concentrations together; canonical methods, except approach **(3)** listed above, require individual analyses for the concentration and the isotope ratio.

Overall, the limits of detection and quantification for NH_4^+ using this method were approximately 0.03 µmol and 0.09 µmol, respectively, and these values are equivalent to 6 µmol/L and 18 µmol/L, if 5 mL of the sample solution (i.e., the sediment extract in this case) is used for the diffusion procedure as described in this paper. Even though using the colorimetric method is recommended to determine the NH_4^+ concentration of samples that have low NH_4^+, the proposed method effectively determines the NH_4^+ isotope ratio and the concentration in samples with relatively high concentrations of ammonium.

ACKNOWLEDGMENTS:

We thank Naoto Tanaka for helping data collection and developing the protocol. The collection of samples was supported by JSPS KAKENHI Grant Number 17K15286.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Gruber, N., Galloway, J.N. An Earth-system perspective of the global nitrogen cycle. *Nature.* **451** (7176), 293-296 (2008).

2 Galloway, J.N. et al. The Nitrogen Cascade. *Bioscience.* **53** (4), 341-356 (2003).

3 Rysgaard S., Risgaard-Petersen, N., Sloth N.P. Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. In: Caumette, P., Castel J., Herbert R. (eds.) *Coastal Lagoon Eutrophication and Anaerobic Processes (C.L.E.AN.)*. Developments in Hydrobiology, vol 117. Springer, Dordrecht, 133-141 (1996).

4 Christensen, P.B., Rysgaard, S., Sloth, N.P., Dalsgaard, T., Schwærter, S. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. *Aquatic Microbial Ecology.* **21**, 73-84 (2000).

5 Tobias, C. R., Anderson, I. C., Canuel, E. A., Macko, S. A. Nitrogen cycling through a fringing marsh-aquifer ecotone. *Marine Ecology Progress Series.* **210**, 25-39 (2001).

6 An, S.M., Gardner, W.S. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). *Marine Ecology Progress Series.* **237**, 41-50 (2002).

7 Gardner, W.S. et al. Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. *Limnology & Oceanography.* **51** (1), 558-568 (2006).

8 Preisler, A. et al. Biological and chemical sulfide oxidation in a Beggiatoa inhabited marine sediment. *The ISME Journal.* **1** (4), 341-353 (2007).

9 Gardner, W.S., McCarthy, M.J. Nitrogen dynamics at the sediment-water interface in shallow, sub-tropical Florida Bay: why denitrification efficiency may decrease with increased eutrophication. *Biogeochemistry.* **95** (2-3), 185-198 (2009).

10 Dong, L.F. et al. Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne estuary, United Kingdom). *Applied and Environmental Microbiology.* **75** (10), 3171-3179 (2009).

11 Koop-Jakobsen, K., Giblin, A.E. The effect of increased nitrate loading on nitrate reduction via denitrification and DNRA in salt marsh sediments. *Limnology & Oceanography.* **55** (2), 789-802 (2010).

12 Dong, L. F. et al. Dissimilatory reduction of nitrate to ammonium, not denitrification or anammox, dominates benthic nitrate reduction in tropical estuaries. *Limnology & Oceanography.* **56** (1), 279-291 (2011).

13 Fernandes, S.O., Bonin, P.C., Michotey, V.D., Garcia, N., LokaBharathi, P.A. Nitrogen-limited mangrove ecosystems conserve N through dissimilatory nitrate reduction to ammonium. *Scientific Reports.* **2**, 419 (2012).

14 Behrendt, A., de Beer, D., Stief, P. Vertical activity distribution of dissimilatory nitrate reduction in coastal marine sediments. *Biogeosciences.* **10** (11), 7509-7523 (2013).

15 Song, G.D., Liu, S.M., Marchant, H., Kuypers, M.M.M., Lavik, G. Anammox, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment. *Biogeosciences.* **10** (11), 6851-6864 (2013).

16 Yin, G., Hou, L., Liu, M., Liu, Z., Gardner, W.S. A novel membrane inlet mass spectrometer method to measure $^{15}NH_4^{+15+}$ for isotope-enrichment experiments in aquatic ecosystems. *Environmental Science & Technology.* **48** (16), 9555-9562 (2014).

17 Zheng, Y. et al. Tidal pumping facilitates dissimilatory nitrate reduction in intertidal marshes. *Scientific Reports.* **6**, 21338 (2016).

18 Bu, C. et al. Dissimilatory Nitrate Reduction to Ammonium in the Yellow River Estuary: Rates, Abundance, and Community Diversity. *Scientific Reports.* **7**, 6830 (2017).

19 Scott, J.T., McCarthy, M.J., Gardner, W.S., Doyle, R.D. Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland. *Biogeochemistry.* **87** (1), 99-111 (2008).

20 Shan, J. et al. Dissimilatory Nitrate Reduction Processes in Typical Chinese Paddy Soils: Rates, Relative Contributions, and Influencing Factors. *Environmental Science & Technology.* **50** (18), 9972-9980 (2016).

21 Silver, W.L., Herman, D.J., Firestone, M.K. Dissimilatory nitrate reduction to ammonium in upland tropical forest soils. *Ecology.* **82** (9), 2410-2416 (2001).

22 Holmes, R.M., McClelland, J.W., Sigman, D.M., Fry, B., Peterson, B.J. Measuring $^{15}N–NH_4^+$ in marine, estuarine and fresh waters: An adaption of the ammonia diffusion method for samples with low ammonium concentrations. *Marine Chemistry.* **60** (3-4), 235–243 (1998).

23 Sigman, D.M. et al. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Analytical Chemistry.* **73** (17), 4145-4153 (2001).

24 Weigand, M.A., Foriel, J., Barnett, B., Oleynik, S., Sigman, D.M. Updates to instrumentation and protocols for isotopic analysis of nitrate by the denitrifier method. *Rapid Communications in Mass Spectrometry.* **30** (12), 1365-1383 (2016).

25 Isobe, K. et al. Analytical techniques for quantifying $^{15}N/^{14}N$ of nitrate, nitrite, total dissolved nitrogen and ammonium in environmental samples using a gas chromatograph equipped with a quadrupole mass spectrometer. *Microbes and Environments.* **26** (1), 46-53 (2011).

26 Miyajima, T., Tanaka, Y., Koile, Y. Determining ^{15}N enrichment of dissolved organic nitrogen in environmental waters by gas chromatography/negative-ion chemical ionization mass spectrometry. *Limnology and Oceanography, Methods.* **3** (3), 164-173 (2005).

27 Stevens, R.J., Laughlin, R.J., Burns, L.C., Arah, J.R.M., Hood, R.C. Measuring the contributions of nitrification and denitrification to the flux of nitrous oxide from soil. *Soil Biology and Biochemistry.* **29** (2), 139-151 (1997).

28 Porubsky, W.P., Velasquez, L.E., Joye, S.B. Nutrient-replete benthic microalgae as a source of dissolved organic carbon to coastal waters. *Estuaries and Coasts.* **31** (5), 860-876 (2008).

29 Huygens, D. et al. Mechanisms for retention of bioavailable nitrogen in volcanic rainforest soils. *Nature Geoscience.* **1** (8), 543-548 (2008).

30 Rutting, T., Boeckx, P., Muller, C., Klemedtsson, L. Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. *Biogeosciences.* **8** (7), 1779-1791 (2011).

31 Song, B., Lisa, J.A., Tobias, C.R. Linking DNRA community structure and activity in a shallow lagoonal estuarine system. *Frontiers in Microbiology.* **5** 460 (2014).

32 Cheng, L. et al. Dissimilatory nitrate reduction processes in sediments of urban river networks: Spatiotemporal variations and environmental implications. *Environmental Pollution.* **219**, 545-554 (2016).

33 Lisa, J.A., Song, B., Tobias, C.R., Hines, D.E. Genetic and biogeochemical investigation of sedimentary nitrogen cycling communities responding to tidal and seasonal dynamics in Cape Fear River Estuary. *Estuarine, Coastal and Shelf Science.* **167** A313-A323 (2015).

34 Deng, F.Y. *et al.* Dissimilatory nitrate reduction processes and associated contribution to nitrogen removal in sediments of the Yangtze Estuary. *Journal of Geophysical Research: Biogeosciences.* **120** (8), 1521-1531 (2015).

35 Tiedje, J. M. In: Zehnder, A. J. B. (ed.) *Biology of Anaerobic Microorganisms*, John Wiley and Sons, 179-244 (1988).

36 Tiedje, J. M., Sexstone, A. J., Myrold, D. D., Robinson, J. A. Denitrification: ecological niches, competition and survival. *Antonie van Leeuwenhoek.* **48** 569-583 (1982).

37 Hardison, A.K., Algar, C.K., Giblin, A.E., Rich, J.J. Influence of organic carbon and nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA) and N_2 production. *Geochimica et Cosmochimica Acta.* **164** (2015).

38 Sigman, D.M. et al. Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: an adaptation of the ammonia diffusion method. *Marine Chemistry.* **57** (3-4), 227-242 (1997).

39 Risgaard-Petersen, N., Rysgaard, S., Revsbech, N.P. Combined microdiffusion-hypobromite oxidation method for determining nitrogen-15 isotope in ammonium. *Soil Science Society of America Journal.* **59** (4) (1995).

40 Gardner, W.S., Bootsma, H.A., Evans, C., John, P.A.S. Improved chromatographic analysis of ^{15}N:^{14}N ratios in ammonium or nitrate for isotope addition experiments. *Marine Chemistry.* **48** (3-4), 271-282 (1995).

Figure 1

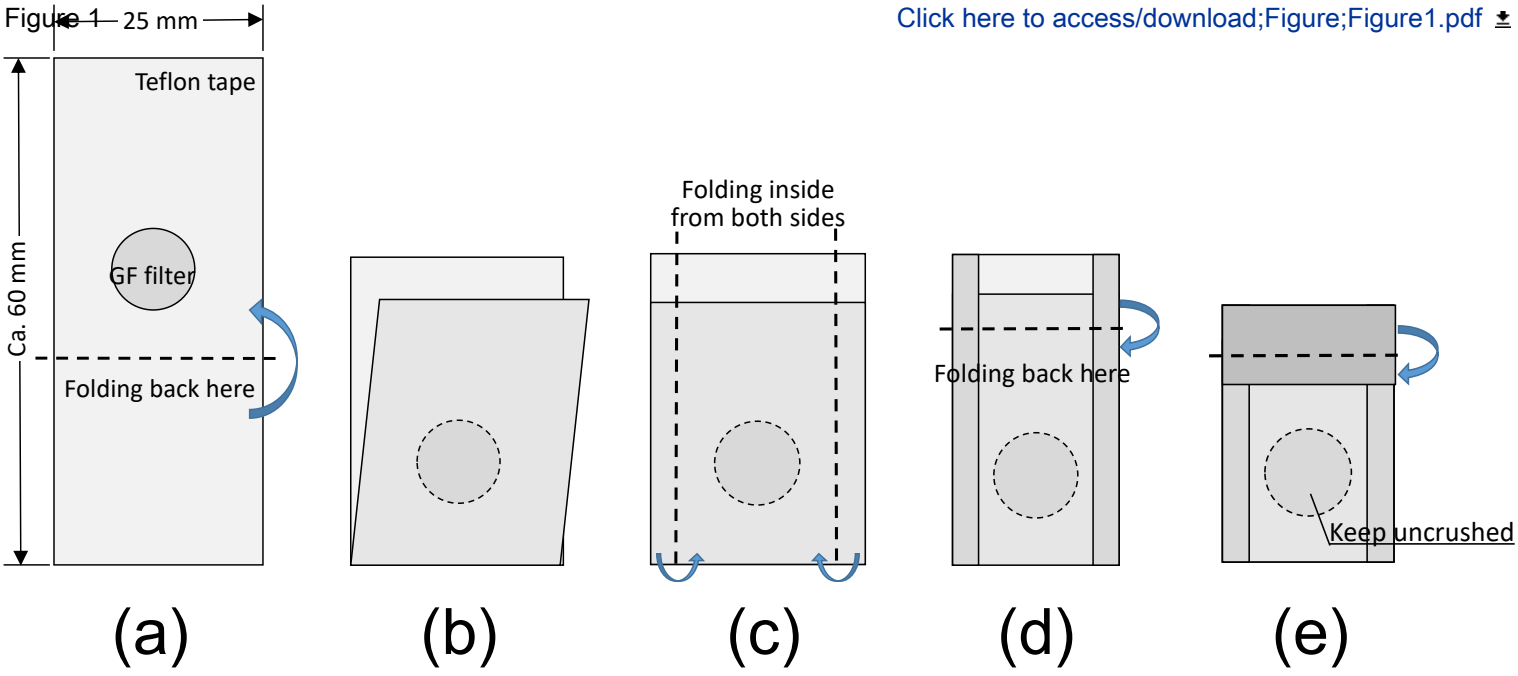

Figure 2

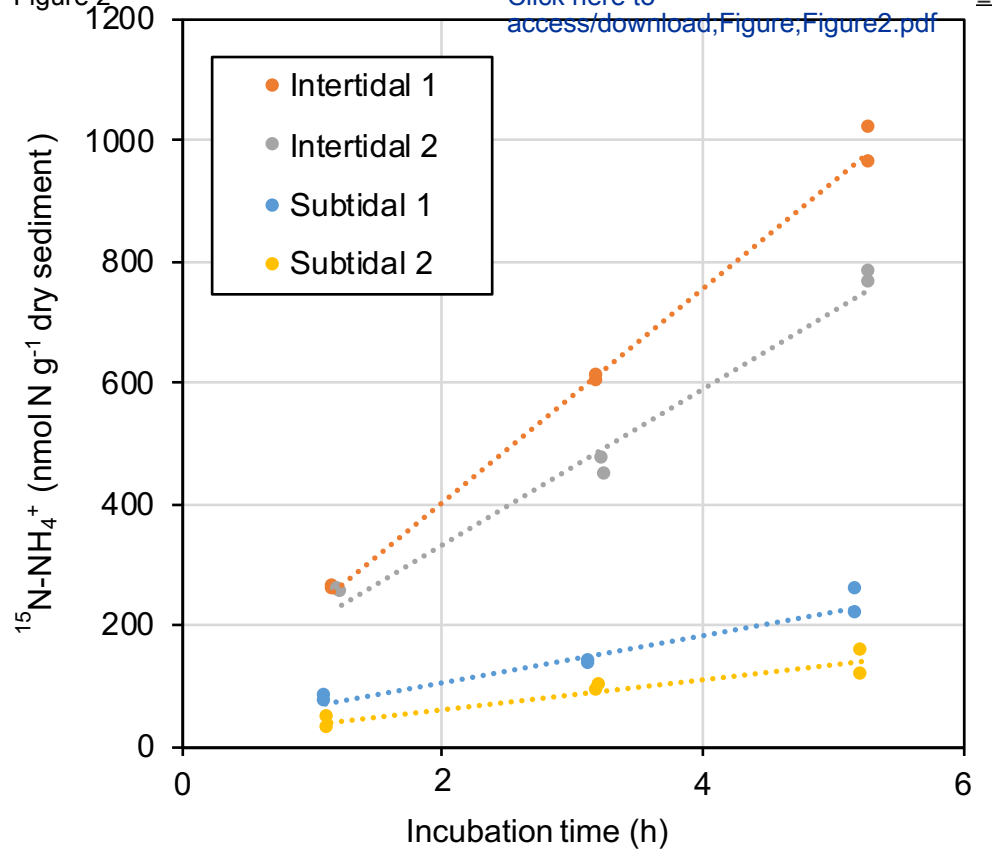

Figure 3

Click here to access/download;Figure;Figure3.pdf

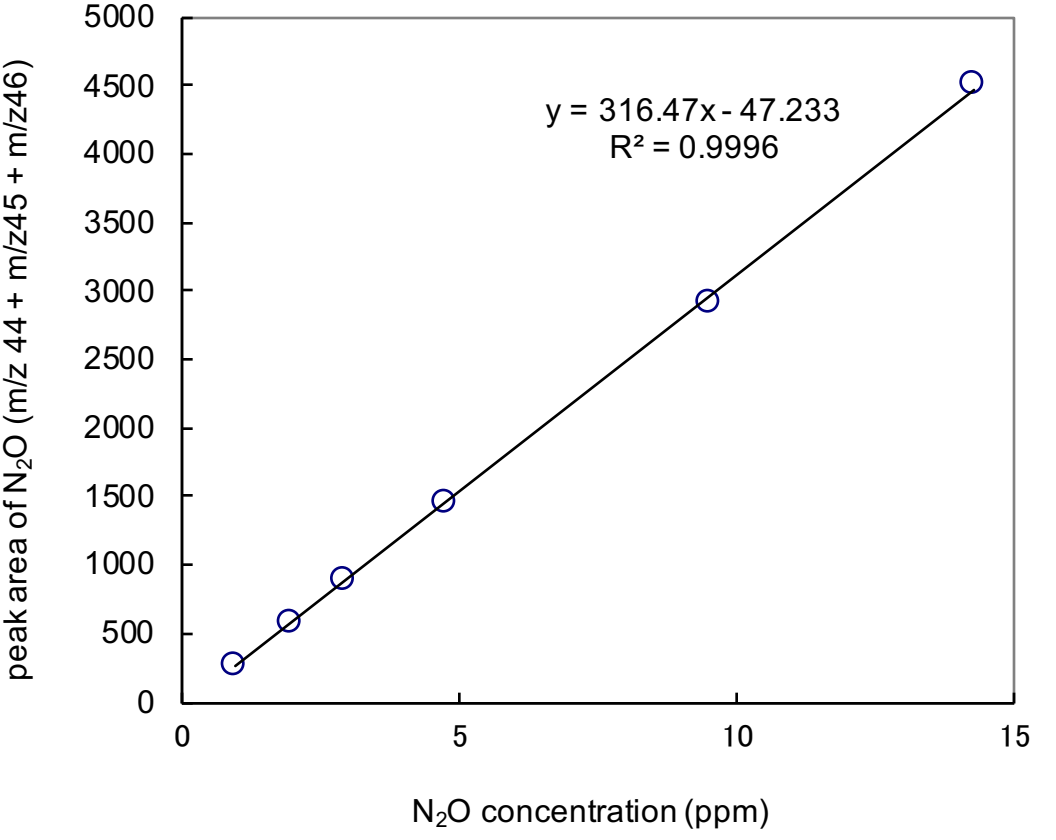

Table 1

	^{15}N-labeled and non-labeled substrates added to each vial	
	100mM NH_4Cl	100mM $K^{15}NO_3$
Volume (µL) of 100 mM stock solution added to each vial	24	60
Final concentration (µmol/L)*	>230§	570

*shown values are calculated by assuming that water content of the sediment is 50%
§depending on the background ammonium concentration

Equipment	
quadrupole GCMS	Shimadzu GCMS-QP2010 Ultra
column	CP-PoraBONDQ 25m; φ 0.32mm; film thickness, 5μm
Analytical conditions	
column temp	40 °C
injection port temp	100 °C
carrier gas stream	Total flow rate, 47.1 mL·min^{-1} flow rate in column, 2.10 mL·min^{-1}
sprit ratio	20
detection voltage	1.5 kv
Sensitivity of N_2O	
lower limit of detection (LOD)*	1.42 pmol
lower limit of quantification (LOQ)*	4.58 pmol

*LOD and LOQ were determined by a linear relationship among a serial dilution of N_2O (0.97, 1.94, 2.91, 4.75, 9.50, 14.3 ppm) in He, corresponding responses in peak area, and S/N ratio. LOD and LOQ were calculated as concentrations equivalent to S/N=3 and S/N=10, respectively.

Table 3

sediment	DNRA rate	enrichment of NO_3^- *
	nmol-N g^{-1} hr^{-1}	atom%
Intertidal 1	177	99.9
Intertidal 2	129	99.0
Subtidal 1	39.3	99.9
Subtidal 2	24.8	99.0

*same as atom% of added KNO_3; complete elimination and no nitrification under used incubation conditions was tested previously.

Name of Material/ Equipment	Company
^{15}N-KNO_3	SHOKO SCIENCE
^{15}N-NH_4Cl	SHOKO SCIENCE
20 mL PP bottle	SANPLATEC
Aluminum cap	Maruemu
Boric acid	Wako
Centrifuge	HITACHI
Deoxygenized Gas Pressure & Repl	SANSIN INDUSTRIAL
Disposable cellulose acetate memb	ADVANTEC
Disposable syringe	Termo
Disposable syringe	Termo
Dulbecco's Phosphate Buffered Sal	NISSUI PHARMACEU
Gastight syringe	VICI Valco Instrume
GC/MS	shimadzu
GF/D	Whatman
Glass vial	Maruemu
Gray butyl rubber stopper	Maruemu
H_2SO_4	Wako
$K_2S_2O_8$	Wako
KCl	Wako
KNO_3	Wako
NaOH	Wako
NH_4Cl	Wako
Plastic centrifuge tube	ASONE
Pseudomonas chlororaphis subsp. a	American Type Cult
PTFE sealing tape	Sigma-Aldrich
Reciprocating shaker	TAITEC
Screw-cap test tube	IWAKI
PTFE-lined cap for test tube	IWAKI
Tryptic Soy Broth	Difco Laboratories

Catalog Number	Comments/Description
N15-0197	
N15-0034	
61-3210-18	Wide-mouth
1307-13	No. 20, with hole
021-02195	
Himac CR21G II	
IP-12	
25CS020AS	Pore size 0.22 μm, 25 mm in diamete
SS-10SZ	10 mL
SS-01T	1 mL
5913	
4075-15010	Series A-2, 100 μL
GCMS-QP2010ultra	
1823-010	10 mm in diameter
0501-06	20 mL
1306-03	No.20-S
192-04696	Guaranteed Reagent
169-11891	Nitrogen and Phosphorus analysis gra
163-03545	Guaranteed Reagent
160-04035	Guaranteed Reagent
191-08625	Nitrogen compounds analysis grade
017-02995	Guaranteed Reagent
1-3500-22	50 mL, VIO-50BN
ATCC 13985	Freeze-dried, the type strain of Pseuc
Z221880	25 mm in width
0000207-000	NR-10
84-0252	11 mL
84-0262	
211825	

r

ade

domonas aureofaciens

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Measurement of the potential rates of dissimilatory nitrate reduction to ammonium (DNRA) based on $^{14}NH_4^+/^{15}NH_4^+$ analysis by sequential conversion and detection with quadrupole GC/MS
Author(s):	Megumi Kuroiwa, Keitaro Fukushima, Kazuma Hashimoto, Yukiko Senga, Tsubasa Sato, Chie Katsuyama, Yuichi Suwa

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Megumi Kuroiwa
Department:	Biological Sciences
Institution:	Chuo University
Title:	Measurement of the potential rates of dissimilatory nitrate reduction to ammonium (DNRA) based on $^{14}NH_4^+/^{15}NH_4^+$ analysis by sequential conversion and detection with quadrupole GC/MS
Signature:	Megumi Kuroiwa
Date:	Dec 14, 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Dr. Steindel

We appreciate all of the valuable comments from you and reviewers on our manuscript, JoVE59562. We have revised the manuscript by carefully addressing the comments, suggestions, and questions. We believe the clarity of our manuscript is greatly improved because of these changes. Please find our detailed response to all comments below; the comments are in italics. Although it is not pointed out by editor and reviewers, we also modified the title of our manuscript, because it exceeded 150-character and used an abbreviation. We apologize that the confirmation before submitting is insufficient. Included in the revised submission is a Word file with changes visible in Track Changes. A version with changes accepted is also included.

Sincerely,
Megumi Kuroiwa

Editor:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

ANSWER：Thank you for your suggestion and we are sorry for our poor English. As the editor advised, we have checked our manuscript and made revisions in terms of language and grammar.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript (and Table 2) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Sigma-Aldrich, Whatman, Nissui Pharmaceutical, Maruemu, Kanda, Wako, Termo,

ANSWER： We deleted the commercial languages and company names from the protocol section to meet the journal requirement. However, we'd like to leave the details of equipment (i.e. model of the GC/MS and column, Table2), since the properties such as separation of gases via column and limit of detection/quantification are largely depend on both the equipment itself and the measurement conditions. We are concerned about lack of these information may lead to misunderstandings. As the editor commented, we modified the Table of Materials so that all commercial products are sufficiently included in the list.

Protocol:

1. Please ensure that all text in the protocol section is written in the imperative tense as if telling

someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible.
ANSWER: As the editor pointed out, we have made some sentence reconstructions to be in the imperative tense from a passive voice.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
ANSWER: We apologize for our carelessness. We have highlighted the essential steps for video filming.

3. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
ANSWER: We have revised the protocol section to meet the journal requirement.

Specific Protocol steps:
1. 1.2: Please explain the ashing procedure further or include a reference.
ANSWER: We have added a sentence for further explanation for ashing process to 1.1.2..

2. 2.1: Where do P. chlororaphis colonies come from?
ANSWER: We have added a description about incubation of *P. chlororaphis* on agar plate as step 2.2.1. in the protocol section.

Figures:
1. Figure 2: Please use 'h', not 'hr'.
ANSWER: Addressed.

Discussion:
1. Please discuss critical steps in the protocol as well as limitations of the protocol in the Discussion section.
ANSWER: We discussed our novelty and validity in line 375-401(changes accepted version). This

part includes the description of our procedures modified from previous methods. We mentioned the limitation of our protocol in line 403-409(changes accepted version).

References:

1. Please ensure references have a consistent format. Please do not abbreviate journal titles.

ANSWER：We have checked reference list and modified to meet a consistent format.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

ANSWER：We checked and modified the list of Table of Materials so that all commercial products/equipments are sufficiently included in the list.

Reviewer #1:

Manuscript Summary:

Excellent idea but need more data from GC MS analysis

ANSWER：We appreciate for your positive comment and advise.

Major Concerns:

One major concern is I need to see the GC MS data. You have mentioned in line 382 that N2O can be measured in GCMS without any atmospheric interference. Can you back that up with the MS data ?

ANSWER：Under the condition shown in table 2, CO_2 and N_2O are completely separated by GC; the retention time of these gases are 1.15 and 1.07 min. respectively. In order to demonstrate that N_2O can be measured with negligible air interference even in low concentrations, we provide an example of calibration curve of low concentration N_2O (Fig. 1, in this document). Since atmospheric concentration of N_2O is on the order of ppb, the influence on the quantitative determination of N_2O due to atmospheric contamination associated with measurement is very limited. The calibration curve passes almost through the origin, demonstrating the influence on the concentration of N_2O due to atmospheric contamination is negligible (Fig.1).

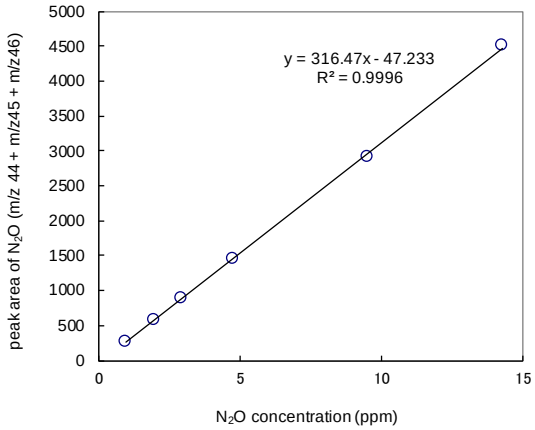

Fig. 1 The relation between N_2O concentration (ppm) and GC/MS peak area. Peak area of N_2O was obtained by sum of the peak area of m/z44, m/z45, and m/z 46.

Reviewer #2:

Manuscript Summary:

The method described will be useful to the community interested in DNRA. I am sure it will be of value to the community of researchers interested in comprehensively addressing the entire N-cycle in various ecosystems. Of particular interest is the potential to increase sensitivity by quantifying N2O instead of ammonium directly. Although this approach requires a series of steps the details are provided very clearly.

ANSWER：We appreciate for your positive comments for significance of our paper.

The Major Concerns:

The most serious issue with this manuscript is the poor quality of the english writing. Frankly it made it difficult to read at times. The detailed methods, however, were sufficient written to make them clear. The remaining sections require significant editing.

ANSWER：We apologize the poor quality of our English. We have used English language editing service and we have made modifications to the manuscript in terms of language and grammar. We have also made some sentence reconstructions and word choice changes for accuracy and enhanced clarity. We believe that these revisions improve readability of our manuscript.