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Preliminary insights into $\delta^{15}N$ and $\delta^{18}O$ of nitrate in natural mosses: A new application of the denitrifier method

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ABSTRACT

Natural mosses have been employed as reactive and accumulative indicators of atmospheric pollutants. Using the denitrifier method, the concentration, $\delta^{15}N$ and $\delta^{18}O$ of moss nitrate (NO_3^-) were measured to elucidate the sources of NO_3^- trapped in natural mosses. Oven drying at 55–70 °C, not lyophilization, was recommended to dry mosses for NO_3^- analyses. An investigation from urban to mountain sites in western Tokyo suggested that moss [NO_3^-] can respond to NO_3^- availability in different habitats. NO_3^- in terricolous mosses showed isotopic ratios as close to those of soil NO_3^-, reflecting the utilization of soil NO_3^-. Isotopic signatures of NO_3^- in corticolous and epilithic mosses elucidated atmospheric NO_3^- sources and strength from the urban (vehicle NO_x emission) to mountain area (wet-deposition NO_3^-). However, mechanisms and isotopic effects of moss NO_3^- utilization must be further verified to enable the application of moss NO_3^- isotopes for source identification.

1. Introduction

Human activities have greatly increased the abundance of reactive nitrogen (N) in the atmosphere, which is responsible for elevated N deposition into ecosystems as well as other environmental consequences (Morin et al., 2008; Bobbink et al., 2010). In recent years, substantial progress has been made in biologically indicating and monitoring approaches of atmospheric N deposition, which greatly complement physical monitoring by providing integrated (time-integrated and N species-integrated), site-based information related to atmospheric N concentrations, N deposition, and ecological impacts (e.g., Pitcairn et al., 2003; Sutton et al., 2007). They have also enabled high-resolution risk assessment (e.g., Zechmeister et al., 2008; Harmens et al., 2011). In parallel, stable isotopic signatures in some potential indicators have been developed to address the source identification of anthropogenic N (Ammann et al., 1999; Pearson et al., 2000; Liu et al., 2008), and evaluate the composition of N deposition altered by human activities (Bragazza et al., 2005; Solga et al., 2005; Xiao et al., 2010).

Nitrate-N (NO_3^- – N) is a dominant component in atmospheric N deposition. The strength and sources of NO_3^- – N in deposition are important aspects of environmental monitoring and ecosystem N biogeochemical studies (Fenn et al., 2003; Elliott et al., 2007). Atmospheric NO_3^- derives mainly from the conversion of nitrogen oxide (NO_x) emissions. Both NO_3^- and NO_x can be deposited directly into ecosystems as dry (gaseous and particulate) and wet forms. Dual N and oxygen (O) isotopes of NO_3^- ($\delta^{15}N$-NO_3^- and $\delta^{18}O$-NO_3^-) have been widely adopted in constraining the source and movement of NO_3^- in various environments (Kendall et al., 2007; Elliott et al., 2009). However, site-specific isotope information of atmospheric NO_3^- is limited by difficulties hindering direct measurement and synthesis of isotopic values of NO_3–N species in both dry and wet deposition. Consequently, isotopic measurements of NO_3^- in plant tissues (plant NO_3^- thereafter), especially for some suitable species such as mosses and lichens, might constitute a conceptual and methodological innovation in bio-monitoring of atmospheric NO_3^-. Moreover, isotope parameters of plant NO_3^- (particularly $\delta^{18}O$) might be straightforward and advantageous to identify the direct incorporation of atmospheric NO_3^- into natural plants, which would benefit a more accurate and quicker evaluation of atmospheric NO_3^- contributions to vegetation of forested ecosystems. For example, $\delta^{18}O$-NO_3^- data are quite useful information for identifying the inputs of atmospheric NO_3^- because of the distinctly higher $\delta^{18}O$ signatures than those of soil NO_3^- (Kendall et al., 2007; Elliott et al., 2009).

Nevertheless, many difficulties exist in measuring stable isotopes of NO_3^- in natural plants. Most natural plants (particularly in N-poor sites) have [NO_3^-] as low as 0.01–1 μmol-N g dry wt^{-1},

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which requires much higher analytical sensitivity than that provided by existing techniques applied for crops and fertilized plants (e.g. 1–117 μmol-N/g dry wt^{-1} in winter wheat; Raun and Westerman, 1991). Traditional flow-injection analysis (FIA), including the pre-reduction of NO_3^- to NO_2^- using copperized cadmium (Cd) or ion chromatography (IC), can only be used for much higher [NO_3^-] in fertilized plants' leaves (>70 μmol-N g dry wt^{-1}) (Anderson and Case, 1999). Moreover, low [NO_3^-] in plant extracts cannot be measured correctly using FIA or IC because organic species in the extracts inactivate the reduction or the chromatography columns (Alves et al., 2000). Particularly, the high concentration of dissolved organic carbon (DOC) in the extracts can severely affect the baseline of IC, thereby reducing the analytical precision of [NO_3^-] measurements. In FIA, DOC can react quickly with the reduced NO_2^- and reduce the efficiency of NO_3^- conversion by clogging the Cd column in FIA, each of which can underestimate the [NO_3^-] (Anderson and Case, 1999). Therefore, conventional techniques are required to remove pigments or other organic compounds completely from plant extracts for the determination of [NO_3^-] (Alves et al., 2000). To date, common methods (e.g., activated carbon, XAD-type resins, ultrafiltration) have been insufficient to remove all organics while leaving NO_3^- intact (Silva et al., 2000). The use of polyvinylpyrrolidone was shown recently to achieve removal in environmental solutions and maintain the isotopic integrity of NO_3^- (Haberhauer and Blochberger, 1999; Michalski, 2010). Nevertheless, no investigation has been conducted on the applicability of this method for isotopic analysis of NO_3^- in plant extracts. The multistep cleanup procedure might present a risk of NO_3^- contamination for low [NO_3^-] (<1 μmol-N g dry wt^{-1}) in high DOC matrices of natural plant extracts.

For NO_3^- isotopes, the N amount required for the conventional Kjeldahl distillation method using isotope-ratio mass spectrometry (IRMS) with dual-inlet measurement is too large (e.g. 70 μmol N; Mulvaney, 1993) to explore the isotopic compositions of NO_3^- in natural plants. Although the combination of the $AgNO_3$ method (Chang et al., 1999; Silva et al., 2000) with IRMS coupled with elemental analyzer (EA-IRMS) or pyrolysis (TC/EA-IRMS) has greatly reduced the sample size for $\delta^{15}N$ and $\delta^{18}O$ measurements to several μmol N (e.g. Xue et al., 2010), the expected [NO_3^-] in natural plants of less than 1 μmol-N g dry wt^{-1} is too low to apply the $AgNO_3$ method. Moreover, the $\delta^{18}O$ measurement of plant NO_3^- can be greatly impaired by DOC contamination of $AgNO_3$ salt. Not only is it extremely difficult to remove the DOC from $AgNO_3$ salt, but the incorporation of a minuscule amount of DOC can strongly affect the $\delta^{18}O$ of NO_3^- measured as CO with TC/EA-IRMS (Michalski, 2010). For the reasons described above, the present study explored the application of the denitrifier method for $\delta^{15}N$ and $\delta^{18}O$ analyses of NO_3^- (Sigman et al., 2001; Casciotti et al., 2002; Koba et al., 2010) in intact extracts of natural plants. The use of the denitrifier for measuring low [NO_3^-], which started during the mid-1980s (Lensi et al., 1985), enables precise measurements of subnanomole-N because of its high sensitivity (Christensen and Tiedje, 1988; Binnerup and Sørensen, 1992). Before that time, Lowe and Hamilton (1967) examined soybean nodule bacteroides for reduction of NO_3^- to NO_2^-, which revealed no interference of DOC on the bacterial conversion of NO_3^- in soil and plant extracts. Because of its high sensitivity, the denitrifier method now enables precise measurements of both $\delta^{15}N$ and $\delta^{18}O$ with 20–50 nmol NO_3^- (Sigman et al., 2001; Casciotti et al., 2002). It is especially applicable for samples with low [NO_3^-] but with high DOC such as the pore water of sediments (Aakra et al., 2000), soil, and plant extracts (e.g., Koba et al., 2010; Liu et al., under review).

Previously, the effect of extraction variables was examined adequately by Anderson and Case (1999), who recommended the extraction of plant NO_3^- with deionized water at room temperature because NO_3^- is readily water-soluble and extraction with water rather than chemical solvents can minimize the contamination of organics. However, plant NO_3^- analysis potentially suffers from the fact that tissue NO_3^- is labile because of the nitrate reductase activity (NRA) in living plant tissues. Much of the work done to date has used markedly different plant drying temperatures (e.g., freeze-drying at −80 °C by Gebauer et al., 1984, oven drying at 60 °C by MacKown and Weik, 2004, and at 105 °C by Cárdenas-Navarro et al., 1999). It remains uncertain if different drying temperatures can efficiently terminate NRA and produce identical [NO_3^-] and isotopic signatures. Accordingly, one primary objective of the present study was to examine the effects of drying temperatures on tissue NO_3^- at the concentration level of natural plants. There were important reasons to select moss as a model for testing the method and explore isotopic signatures of NO_3^- in natural mosses. First, mosses are functional communities playing important roles in various natural and affected ecosystems (Turetsky, 2003; Wanek and Pörtl, 2008). Secondly, moss bulk $\delta^{15}N$ showed values close to that of anthropogenic NO_3^- sources (Pearson et al., 2000), and no substantial isotopic fractionation was assumed during the influx of N to moss cells (Bragazza et al., 2005). Therefore, $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- trapped in natural mosses might provide unique insights into atmospheric NO_3^- sources. Theoretically, plant NR would cause the reduction of tissue NO_3^-. Thereby, isotopic ratios of extracted NO_3^- would be more enriched than initial sources (Evans et al., 1996). However, no direct evidence exists from field or experimental studies to demonstrate the extent to which moss NO_3^- isotopes can serve as a proxy of NO_3^- sources. Mosses can trap atmospheric N efficiently because of their large surface area, but their NO_3^- uptake and consumption might be low as a result of the short supply of other nutrients and preferences for ammonium-N and DON (Soares and Pearson, 1997; Wanek and Pörtl, 2008). Based on the reasons described above, our second objective was to elucidate variations of the concentration, $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in natural mosses in response to NO_3^- available in different habitats. Specific hypotheses to be tested were the following: (1) Samples dried at different temperatures present comparative [NO_3^-] and isotope ratios with those being ground and extracted directly. Improper drying does not terminate NR, resulting in lower [NO_3^-] and enriched isotope ratios because of the NO_3^- reduction by NR. (2) Given the apparent isotopic effects that occur during plant NO_3^- assimilation (Ledgard et al., 1985; Tcherkez and Farquhar, 2006), $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in natural mosses would be isotopically enriched compared with their potential sources if a substantial fraction of NO_3^- was assimilated, otherwise moss NO_3^- isotopes could differentiate NO_3^- sources in distinct habitats.

2. Materials and methods

2.1. Moss sampling and treatment

To test the effect of drying temperatures on NO_3^- extraction of the same moss, a sheet of *Hypnum plumaeforme* Wils. (58 cm × 39 cm) was purchased from a gardening company (Moss Farm, http://www.mossfarm.jp, Shizuoka, Japan). It was stored in the low-light environment of the laboratory. Deionized water and N-free macronutrient solution (modified from Alghamdi, 2003) were supplied to the moss every two days. Green tissues of the moss were collected and washed with deionized water to remove adsorbed impurities and pollutants thoroughly. Then excess water was drained off for a blank check. Washed samples were well mixed, weighed, and then dried at room temperature (25 °C), in an oven (40 °C, 55 °C and 70 °C) or in a freeze drier (−53 °C) to constant weights. Then they were finely ground using a ball mill (MM200; Retsch GmbH and Co. KG). The washed moss samples were homogenized directly using a mortar and pestle. Then they were extracted immediately. Temperatures of >70 °C were not tested because oven drying at >60 °C has been found to influence plant $\delta^{15}N$ significantly (e.g. Brearley, 2009). The number (n) of samples varied due to different amount of samples.

Field moss sampling was conducted in western Tokyo, central Japan (Table 1). The study region has a temperate monsoon climate with an annual average

Table 1
Description of sampling sites of natural mosses in western Tokyo area.

Sampling site	Location	Elevation	Traffic intensity	Habitats	Species
Urban	35° 40′N; 139° 28′E	59 m	High	Corticolous	*Leskeella nervosa*
Suburban	35° 38′N; 139° 16′E	216 –223 m	Medium	Epilithic	*Entodon angustifolium* *Entodon longifolius* *Sematophyllum japonicum*
Suburban	35° 38′N; 139° 16′E	223 m	Medium	Terricolous	*Plagiomnium acutum*
Mountain	35° 38′N; 138° 14′E	500 –589 m	Low	Corticolous	*Brachytheciun plumosum* *Leskeella nervosa*

temperature of 15.3 °C. The average annual rainfall is nearly 1500 mm. Natural corticolous mosses were collected at an urban site (Fuchu) and at a mountain site (Mt. Takao), whereas epilithic mosses, terricolous mosses and subsoil (0–10 cm) were sampled at a suburban site (Hachioji, Table 1). All naturally growing mosses were dried at 55 °C and treated as described above.

2.2. Moss NO_3^- extraction and measurement

In 20 ml headspace vials, 0.25 g dried and ground moss samples and 10 ml deionized water were mixed. Then the vials were evacuated in a vacuum desiccator for 30 min to ensure sufficient penetration of water into interstitial spaces after methods for NRA (Koyama and Tokuchi, 2003). All extractions were performed at room temperature (25 °C). The extracts were not filtered. The vials were crimp-sealed with Teflon-backed silicone septa (20-AC-CBT3; Chromacol) before shaking for 30 min. The denitrifier *Pseudomonas aureofaciens* (ATCC# 13985) was incubated for 6–10 days in working medium of Tryptic Soy Broth (Difco) amended with KNO_3, NH_4Cl, and KH_2PO_4 according to the method described by Casciotti et al. (2002) and Koba et al. (2010). Before use, *P. aureofaciens* cells were concentrated by centrifugation, washed with NO_3^--free medium and dispensed into new NO_3^--free medium. This NO_3^--free media with denitrifier was purged with pure N_2 gas for 2 h. The moss extract was purged with pure N_2 gas for 1 h, then 2 ml of the purged NO_3^--free medium with denitrifiers was injected into vials with moss extract using disposable syringes (Terumo Corp.) and needles (26 gauge; Terumo Corp.). The vials were incubated overnight on a horizontal shaker to allow for complete conversion of NO_3^- to N_2O before the addition of 0.2 ml of 5 M NaOH to stop the bacterial activity and to scavenge CO_2. The septa were sealed using silicone sealant (KE-42-T; Shin-Etsu Chemical Co. Ltd.). The vials, which were stood upside down so as to prevent leakage after each injection, were kept at 25 °C to equilibrate the dissolved and headspace N_2O.

Concentrations of N_2O in the headspace of the sample vials were first measured using a gas chromatograph equipped with an electron capture detector (GC/ECD, GC-14B; Shimadzu Corp., Kyoto, Japan). 0.5 ml of the headspace (7.55 ml) of each sample vial was injected to the GC/ECD for N_2O determination. The calibration curve between the measured N_2O (peak area) and $[NO_3^-]$ in extracted solution (μmol N l^{-1}), and $[NO_3^-]$ in plant material (μmol N g dry wt^{-1}) (Fig. 1) was prepared using standards with known $[NO_3^-]$ (Christensen and Tiedje, 1988; Binnerup and Sørensen, 1992; Højberg et al., 1994). The $[NO_3^-]$ in blanks was less than 0.1 μmol N l^{-1} (equivalent to 0.004 μmol N g dry wt^{-1} in 0.25 g plant). The recovery rates of NO_3^- in plant extracts were checked by the spiking method (Table S1 in Supplemental Information), showing an average NO_3^- recovery rate of 100.3 ± 4.5% for our protocol. Moreover, isotopic ratios of NO_3^- spiked into moss extracts were found being consistent with those in deionized water (Table S2 in SI). It can be concluded that the sample preparation and conversion of NO_3^- by the denitrifier were not influenced by the NR and DOC in unfiltered plant extracts.

The apparatus used for isotopic measurement included an isotope-ratio mass spectrometer (Delta XP; Thermo Fisher Scientific Inc., Yokohama, Japan) coupled with Precon (ThermoFinnigan) and GC (Agilent, HP6890, Hewlett Packard Co., Palo Alto, CA, USA) equipped with Poraplot column (25 m × 0.32 mm) and GC interface III (Thermo Fisher Scientific Inc., Yokohama, Japan). After the measurement of $[NO_3^-]$ using GC/ECD, all N_2O in the sample vial was cryofocused using a purge-and-trap system. Then the purified and cryofocused N_2O was introduced into the GC-IRMS. We baked out the column at 175 °C for 2 min after each measurement to eliminate carbon compounds accumulating in the column, which can affect $\delta^{18}O$ analysis (McIlvin and Casciotti, 2011). The calibration curve between measured isotope ratios of N_2O and those of NO_3^- was prepared using USGS-32, USGS-34, USGS-35, and IAEA-NO_3. The natural abundances of ^{15}N and ^{18}O were calculated as $\delta^{15}N$ and $\delta^{18}O$ values expressed in per mil units (‰), as

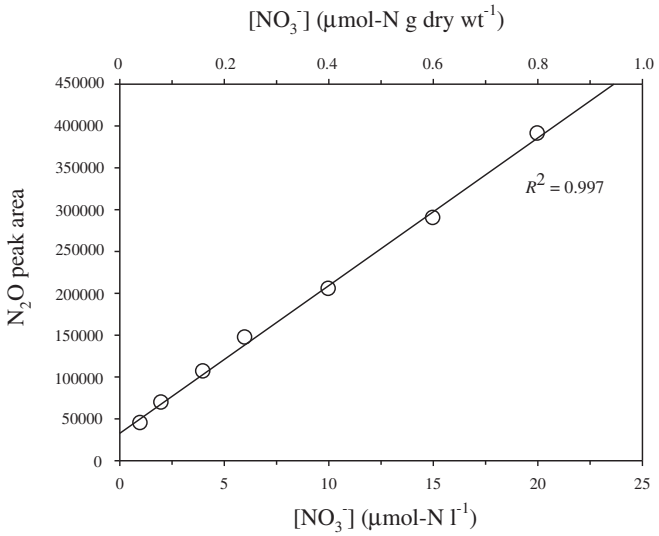

Fig. 1. Correlation between measured N_2O (peak area) produced by NO_3^- standards and corresponding $[NO_3^-]$ in solution (1–20 μmol N l^{-1}), and equivalent $[NO_3^-]$ (0.04–0.80 μmol N g dry wt^{-1}) in 0.25 g plant.

$$\delta^{15}N \text{ and } \delta^{18}O = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000,$$

where $R = {}^{15}N/{}^{14}N$ or $^{18}O/^{16}O$. The analytical precision for $\delta^{15}N$-NO_3^- was better than 0.2‰ and 0.5‰ for $\delta^{18}O$-NO_3^-.

2.3. Soil NO_3^- extraction and measurement

Soils were passed through a 2 mm mesh sieve to remove roots and coarse fragments. Sieved soils were used to determine the water content and were extracted with 2 M KCl solution within 8 h after the sampling. The $[NO_3^-]$ in soil extracts was determined colorimetrically using an autoanalyzer (TRAACS 800; Bran-Luebbe, Tokyo, Japan). The KCl extracts were frozen until isotopic analysis using the denitrifier method described above (Koba et al., 2010).

2.4. Statistics

One-way ANOVA was performed to examine the differences in $[NO_3^-]$, $\delta^{15}N$-NO_3^- and $\delta^{18}O$-NO_3^- among drying temperatures. Paired and independent sample *t*-tests were conducted to determine if the values (means ± SD) were significantly different from each other. Analyses were conducted using software (SPSS 13.0 for Windows; SPSS Inc.). A significant difference between the results was inferred for $P < 0.05$.

3. Results

3.1. $[NO_3^-]$ and isotope ratios of mosses dried at different temperatures

Three discontinuous tests were conducted on *H. plumaeforme* (Fig. 2). Lyophilized (freeze-dried at −53 °C) mosses were occasionally found yielding lower $[NO_3^-]$, more positive $\delta^{15}N$-NO_3^- and/or $\delta^{18}O$-NO_3^- than those of oven dried mosses in two independent tests (tests 1 and 2 in Fig. 2). Mosses dried at 55 °C showed $[NO_3^-]$ and $\delta^{15}N$ values comparable to those that were ground freshly and extracted immediately (test 2 in Fig. 2). The $[NO_3^-]$ did not differ among mosses dried at 40 °C, 55 °C, and 70 °C (Fig. 2c), and NO_3^- isotopes did not differ between mosses dried at 55 °C and 70 °C (Fig. 2f and i).

3.2. $[NO_3^-]$ and isotope ratios of natural mosses in the western Tokyo area

Corticolous mosses at the urban site contained higher $[NO_3^-]$ (0.26 ± 0.04 μmol N g dry wt^{-1}) than those at the mountain sites

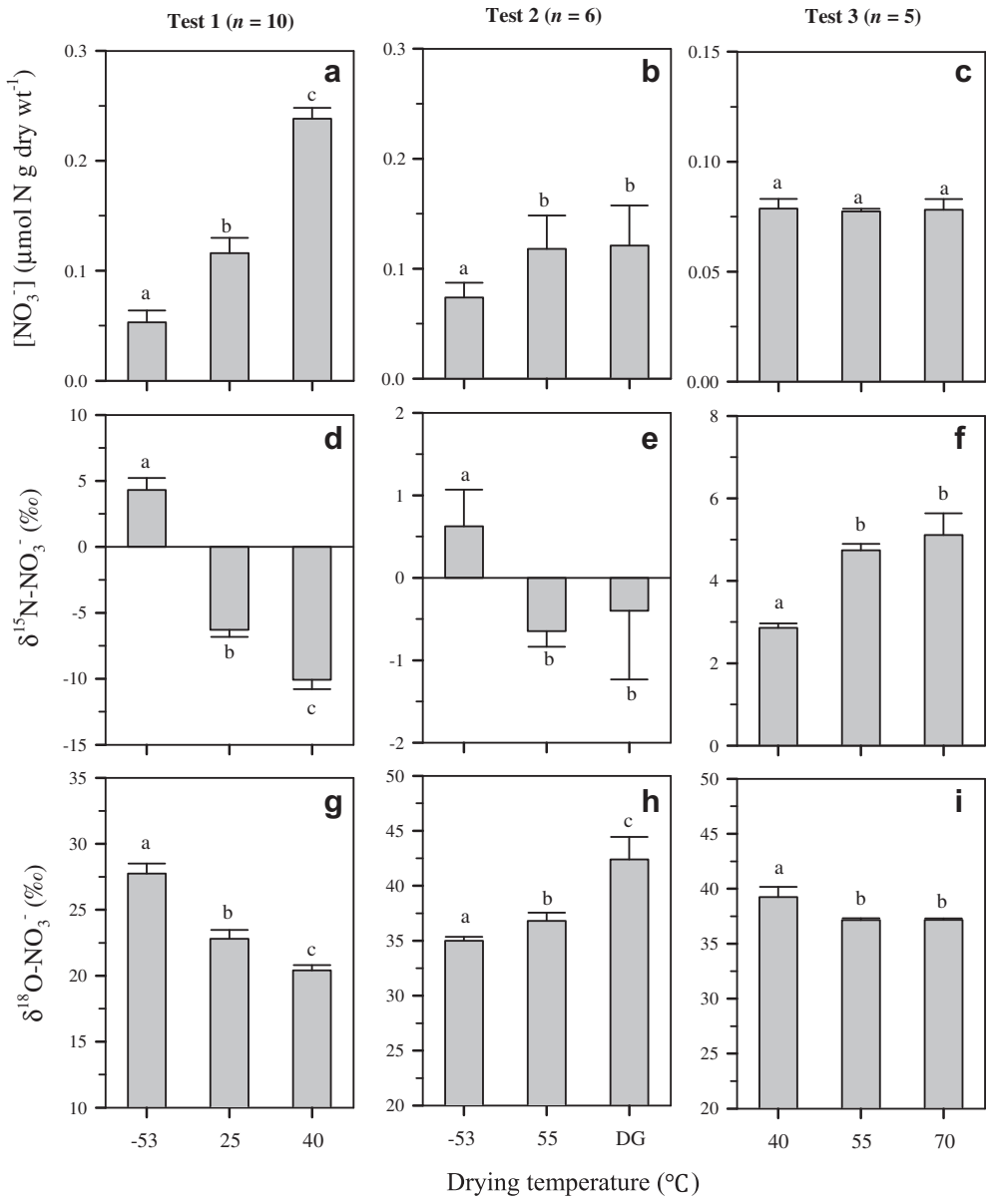

Fig. 2. Concentration, $\delta^{15}N$, and $\delta^{18}O$ of NO_3^- in moss (*H. plumaeforme*) dried at different temperatures. Values (means ± SD) not sharing the same superscript or subscript letters are significantly different in a single panel at $P < 0.05$. DG represents direct grinding of fresh moss tissues.

(0.11 ± 0.01 μmol N g dry wt^{-1}). Terricolous mosses showed higher [NO_3^-] (0.23 ± 0.004 μmol N g dry wt^{-1}) than epilithic mosses (0.11 ± 0.004 μmol N g dry wt^{-1}) collected at the same location (Fig. 3a).

The NO_3^- in terricolous suburban mosses showed isotopic compositions of 1.5 ± 0.4‰ for $\delta^{15}N$ and 23.6 ± 0.4‰ for $\delta^{18}O$, which more closely resemble those of soil NO_3^- (−1.8 ± 0.4‰ for $\delta^{15}N$ and 22.9 ± 1.8‰ for $\delta^{18}O$) when compared with other sources (Fig. 3b and c). Corticolous urban mosses showed the lowest NO_3^- isotope ratios (−7.3 ± 3.6‰ for $\delta^{15}N$ and 18.3 ± 1.9‰ for $\delta^{18}O$). Less negative values were found in epilithic suburban mosses (mean = −5.4‰ to −6.1‰ for $\delta^{15}N$ and 30.0‰–41.4‰ for $\delta^{18}O$). The NO_3^- isotopes of corticolous mountain mosses (mean = 0.4‰–1.7‰ for $\delta^{15}N$ and 40.1‰–45.9‰ for $\delta^{18}O$) were elevated, which showed a trend toward those of wet-deposited NO_3^- (3.0 ± 0.3‰ for $\delta^{15}N$ and 76.9 ± 0.3‰ for $\delta^{18}O$, $n = 3$; Unpublished data) collected in the vicinity in 2009 (FM Tamakyuryo experimental forest of Tokyo University of Agriculture and Technology).

Regarding the data of epilithic and corticolous mosses from the urban to mountain sites, the moss [NO_3^-] showed no clear correlation with $\delta^{15}N$ ($R^2 = 0.19$, $P = 0.05$), but with $\delta^{18}O$ ($R^2 = 0.71$, $P < 0.0001$) (Fig. 4). The spatial distribution of $\delta^{15}N$ and $\delta^{18}O$ signatures of NO_3^- in mosses was generally lowest in urban areas, somewhat higher in suburban areas, and highest at mountain sites (Fig. 5).

4. Discussion

4.1. Influences of different drying temperatures on moss NO_3^-

Compared with those of oven dried mosses, lower [NO_3^-] and higher isotopic signatures in lyophilized mosses (Fig. 2) suggest the occurrence of NO_3^- losses or assimilation with isotopic fractionation in the lyophilized samples. An alternative reason for NO_3^- losses during lyophilization might be sublimation at low pressures and in dry environments, which has been observed in surface snow layers

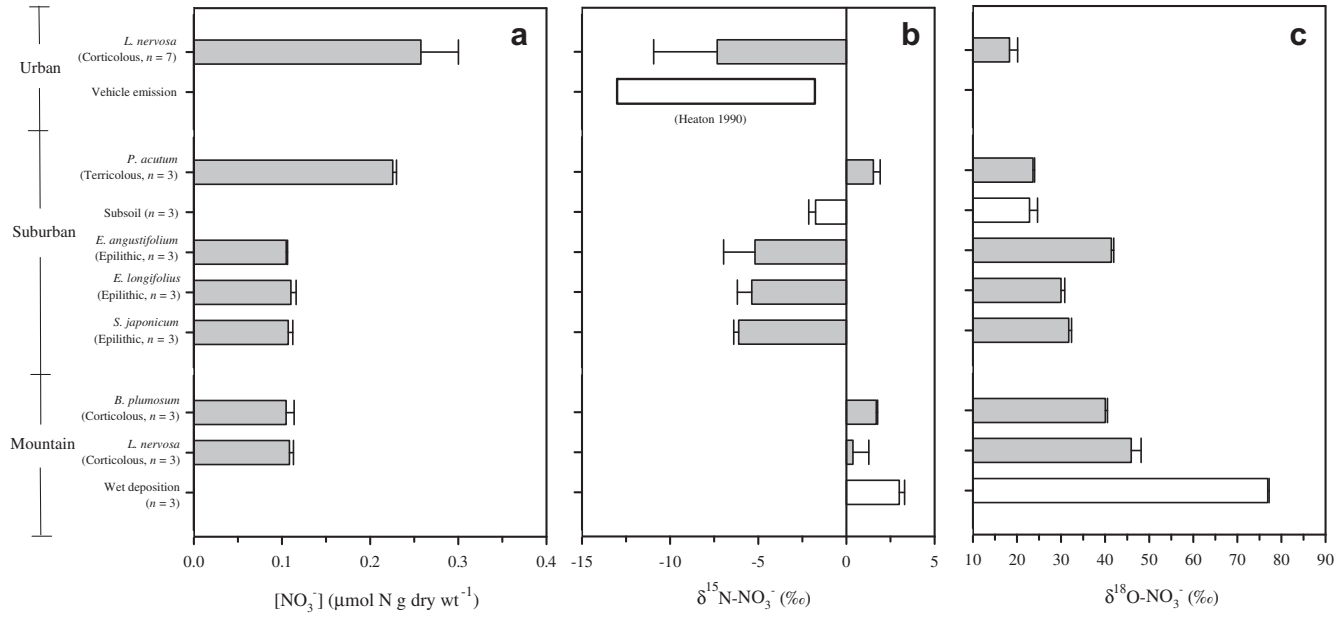

Fig. 3. The $[NO_3^-]$ (a), $\delta^{15}N\text{-}NO_3^-$ (b), and $\delta^{18}O\text{-}NO_3^-$ (c) of natural mosses in the western Tokyo area. Data are expressed as means ± SD of the *n* sample range. Open bars are isotopic signatures of NO_3^- sources in soil of terricolous mosses and wet deposition. The open strip in (b) shows $\delta^{15}N$ data (−13‰ – −2‰) of vehicle NO_x cited from Heaton (1990).

of the Arctic and Antarctic regions (e.g., Nakamura et al., 2000; Heaton et al., 2004; Frey et al., 2009). Furthermore, as reported for the case of *Sphagnum* moss (Woodin and Lee, 1987), high temperatures would be required to inactivate NR (Theodorides and Pearson, 1982). Mosses appeared greenish after lyophilization, which differed from the discolored appearance of oven dried samples. That phenomenon suggests that lyophilization at −53 °C can prevent complete damage to chloroplasts and pigments. The NRA remains active or can be reactivated before extraction. Similarly, results showed that microbiological reduction of NO_3^- can take place when vegetables are stored under refrigeration and deep-freezing (Ezeagu, 1996; Prasad and Chetty, 2008). In contrast, Cabello et al. (1998) reported that damaging chloroplasts in plants was sufficient to abolish NR induction almost completely because NO_2^- reduction occurs in the chloroplasts of green tissues. Our results also reflected the rationale that lyophilization is widely accepted as preserving enzymes, whereas autoclaving at high temperatures is used to kill them.

Significantly lower concentrations and isotopic enrichments of NO_3^- in mosses dried at 25 °C (Fig. 2a–c) showed that NR was also active at moderate oven temperatures. In this study, the yields of NO_3^- did not differ among mosses dried at 40 °C, 55 °C, and 70 °C (Fig. 2g), but another experiment showed that coniferous leaves that had been dried at 40 °C exhibited lower $[NO_3^-]$ with significant $^{15}N\text{-}NO_3^-$ enrichment compared with those dried at either 55 °C or 70 °C (Liu et al. unpublished). Accordingly, 55–70 °C is likely to be safer for drying plant samples for NO_3^- extraction. For measurements of natural mosses, 55 °C was used in this study.

4.2. $[NO_3^-]$ and isotope ratios of natural mosses in response to potential sources

The responsiveness of $[NO_3^-]$ in field plants to elevated NO_3^- availability has been reported rather rarely. Although tissue NO_3^- is labile, the leaf NO_3^- of forest plants has been found to increase with soil N availability. Therefore, it can be an indicator of N pollution and forest N saturation (e.g., Fenn and Poth, 1998; Jones et al., 2008). This study reports the first data of NO_3^- in natural mosses in response to NO_3^- availability. Corticolous mosses at the urban site accumulated more NO_3^- than those at the mountain site (Fig. 3a), which presumably reflected a higher level of atmospheric NO_3^- and/or NO_x in the urban area. Similarly, in the suburban area, higher $[NO_3^-]$ of terricolous mosses than epilithic mosses (Fig. 3a) reflected a higher NO_3^- availability (from both atmosphere and soil) for

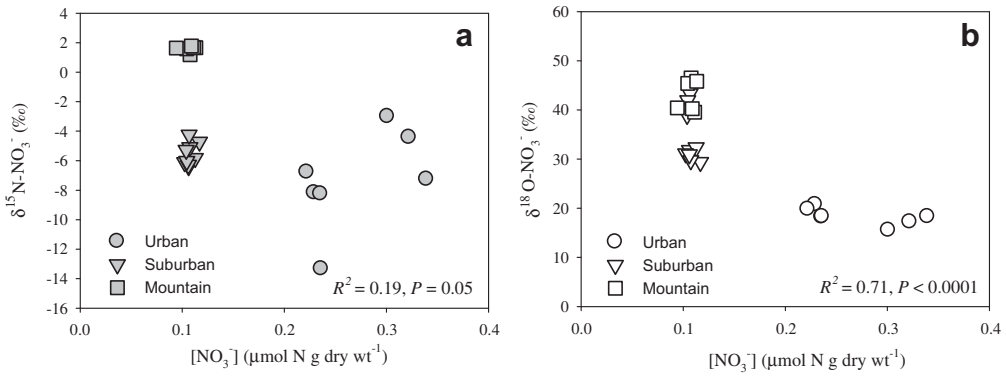

Fig. 4. Correlations between $[NO_3^-]$ and (a) $\delta^{15}N\text{-}NO_3^-$, (b) $\delta^{18}O\text{-}NO_3^-$ of natural mosses (corticolous and epilithic) from urban to mountain sites in the western Tokyo area.

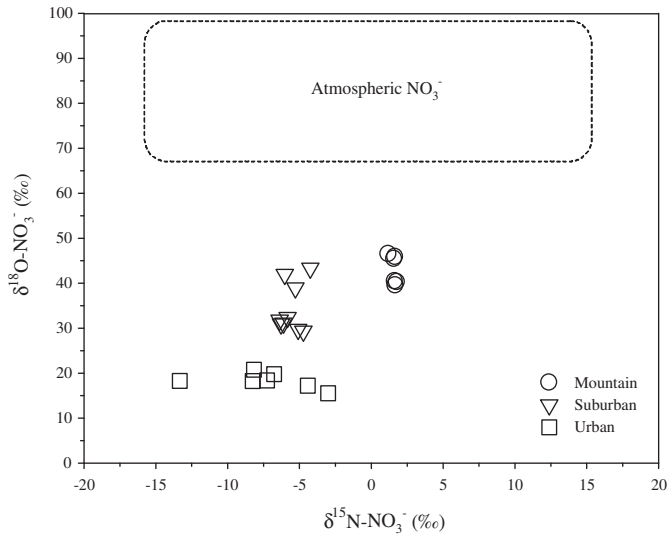

Fig. 5. The $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ distribution of epilithic and corticolous mosses at the urban, suburban, and mountain sites in the western Tokyo area. The data range of atmospheric NO_3^- (rectangles in dashed line) was referred from Kendall et al. (2007) and references therein.

mosses attached to soils. Moreover, the accumulation of NO_3^- in corticolous mosses was not species-dependent, but rather site-specific because 1) *Leskeella nervosa* showed distinct $[NO_3^-]$ between the urban (0.26 ± 0.04 μmol N g dry wt^{-1}) and mountain sites (0.11 ± 0.01 μmol N g dry wt^{-1}), and 2) different species at the same site showed similar $[NO_3^-]$ (Fig. 3a).

The depleted $\delta^{15}N\text{-}NO_3^-$ ratios of urban corticolous mosses (−13.3‰ to −3.0‰, Fig. 3b) resemble those of motor vehicle NO_x emissions (−13‰ to −2‰), as reported by Heaton (1990), and differ distinctly from those from coal-fired power plants (+6‰ to +13‰, Heaton, 1990; Kiga et al., 2000). This result suggests that traffic NO_x emissions constitute the dominant source of NO_3^- in the urban atmosphere. The $\delta^{18}O\text{-}NO_3^-$ values (15.5‰–20.8‰) of urban mosses suggest that urban traffic NO_x might have lower $\delta^{18}O$ signatures than wet-deposited NO_3^- collected distantly from urban areas (Fig. 3c). Mountain mosses showed NO_3^- isotopes that more closely resembled those of wet NO_3^- deposition (Fig. 3b and c), suggesting a source shift from urban-derived NO_x in the urban area (shown by the NO_3^- isotopes of urban corticolous mosses: -7.3 ± 3.6‰ for $\delta^{15}N$ and 18.3 ± 1.9‰ for $\delta^{18}O$) to wet NO_3^- deposition at the mountain site. Suburban mosses expressed NO_3^- isotopic ratios between those of urban and mountain sources (Fig. 3), revealing a mixing of main sources from wet-deposited NO_3^- and urban-derived NO_x.

The uptake of soil NO_3^- by terricolous mosses was supported by tissue NO_3^- isotope ratios resembling those of soil NO_3^- (Fig. 3b and c). Previously, the utilization of soil NO_3^- by terricolous mosses was demonstrated by an experimental study (Van Tooren et al., 1990) and an ^{15}N-tracer study (Ayres et al., 2006). Our study provides new evidence by measuring tissue NO_3^- isotopes in field mosses. Because isotopic signatures of wet-deposited NO_3^- can not represent those of bulk NO_3^- deposition (including wet and dry depositions) trapped by mosses (Heaton et al., 1997), the fractional contributions of soil NO_3^- and atmospheric NO_3^- in terricolous mosses can not be calculated in this study. Moreover, although the isotopic effects of moss NO_3^- uptake were presumably low (Werner and Schmidt, 2002; Bragazza et al., 2005), significant N isotopic fractionation of 15‰ during nitrate reduction has been reported on spinach in an experimental study (Ledgard et al., 1985; Tcherkez and Farquhar, 2006). Further determination or proper evaluation of both tissue NO_3^- kinetics and N/O isotopic fractionations in bryophytes is critical (Wanek and Pörtl, 2008).

4.3. Implications of moss $[NO_3^-]$ and isotopic variations from the urban to mountain area

Plants with higher metabolic capacity (N demand) usually show higher levels of NR and NO_3^- uptake (Imsande and Touraine, 1994). Consequently, they show higher NO_3^- isotope ratios than the sources (Evans et al., 1996), all of which can be enhanced by NO_3^- availability because of the induction of NR by NO_3^- (Tcherkez and Hodges, 2008). However, moss NO_3^- data indicated no substantial isotopic enrichment compared with their potential sources, particularly for urban mosses under higher N availability (Fig. 3). If the decreased moss $[NO_3^-]$ and increased NO_3^- isotopes in mountain mosses had resulted from NO_3^- assimilation, then isotopic ratios of moss NO_3^- would correlate negatively with $[NO_3^-]$. Then ^{15}N and ^{18}O enrichments of NO_3^- would conform to a ~1:1 trend, showing assimilatory isotopic fractionations (Granger et al., 2004). However, moss $[NO_3^-]$ (epilithic and corticolous) showed no clear correlation with $\delta^{15}N$, but with $\delta^{18}O$ from the urban to mountain sites (Fig. 4). Furthermore, in an experimental study of the moss *H. plumaeforme*, the extracted tissue NO_3^- showed isotopic ratios (mean $\delta^{15}N = 1.6 \pm 0.2$‰ and $\delta^{18}O = 24 \pm 0.4$‰, $n = 2$) resembling the supplied NO_3^- ($\delta^{15}N = 1.6 \pm 0.2$‰ and $\delta^{18}O = 26.0 \pm 0.6$‰, $n = 10$) (Liu et al., unpublished data), reflecting insignificant isotopic effects of moss NO_3^- in certain conditions. Accordingly, the spatial pattern of moss $[NO_3^-]$ and isotopic variations might not result from NO_3^- assimilation. Instead, they might be attributed mainly to the decrease of low-$\delta^{18}O$ NO_x and the simultaneous increase of high-$\delta^{18}O$ wet-deposited NO_3^- from the urban to mountain area. Therefore, dual N and O isotopic analyses of NO_3^- in moss tissues provided specific information about the source type and relative contribution of atmospheric NO_3^-.

5. Conclusions

This study applied the denitrifier method to measurements of the concentration, $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in natural moss species, which have long been difficult to obtain using conventional methods. The merits of the denitrifier method and the contribution of this work can be summarized as outlined below.

The applicability of the denitrifier (*P. aureofaciens*) to convert NO_3^- to N_2O in untreated plant extracts and in water-plant homogenates was confirmed. Results clearly showed the absence of a negative effect of DOC on the bacterial reduction of NO_3^- compared with existing chemical methods. Moreover, by obviating filtration, purification, and preconcentration, our protocol greatly minimized NO_3^- loss and contamination from complex sample pretreatments. Because of the high sensitivity of GC-ECD and GC-IRMS, it greatly reduced the amount of plant sample (0.25 g) and the volume of extracts (<10 ml) necessary for concentration and isotopic determinations. The method can detect plant NO_3^- as low as 0.03 μmol N g dry wt^{-1} (found in old moss, unpublished data). For the first time ever reported, $\delta^{15}N$ and $\delta^{18}O$ signatures of NO_3^- in natural plants were measured, and 55–70 °C were recognized as optimal oven temperatures for plant NO_3^- analyses. This information and method constitute important contributions to further efforts at measurements of NO_3^- concentration and isotope ratios in plant samples.

The $[NO_3^-]$ of natural mosses did not differ among species with similar functional types. They depended on site-specific NO_3^- pollution and accessibility of soil NO_3^-. The NO_3^- isotopes of terricolous mosses revealed the utilization of soil NO_3^-. For that reason, mosses growing on soils are not reliable indicators of

atmospheric NO_3^-. NO_3^- in corticolous and epilithic mosses reflected the source shift from the urban traffic NO_x to wet-deposited NO_3^- at mountain sites in the western Tokyo area. Notably, tissue [NO_3^-] and isotopic parameters measurements allow natural mosses to be used as proxies to differentiate atmospheric NO_3^- sources. However, studies of isotopic effects of moss NO_3^- uptake and assimilation are strongly required to advance the application of moss NO_3^- isotopes for elucidating NO_3^- sources and metabolisms.

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Appendix. Supplementary Information

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