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Nitrogen and oxygen isotope effects of tissue nitrate associated with nitrate acquisition and utilisation in the moss *Hypnum plumaeforme*

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Abstract. Mosses are effective accumulators and indicators of N deposition, but the mechanisms of moss N utilisation remain unclear. This study monitored nitrate concentrations ([NO_3^-]) in solutions supplied to *Hypnum plumaeforme* Wils. to characterise NO_3^- uptake from rain events. Concentrations and isotopic ratios ($\delta^{15}N$ and $\delta^{18}O$) of residual NO_3^- in moss tissues were measured to interpret induced NO_3^- reduction. Noninduced NO_3^- reduction was inferred from endogenous [NO_3^-] and isotopic variations that occurred during 65 days of N deprivation. *H. plumaeforme* scavenges NO_3^- effectively from supplied solutions. The uptake rate increased with substrate [NO_3^-] (0.4–3.9 mg N L^{-1}) and generally obeyed saturation (Michaelis–Menten) kinetics. The uptake rate was maximised within 60 min after receiving NO_3^-, irrespective of the initial substrate [NO_3^-]. Lower tissue [NO_3^-] and greater isotopic enrichment verified the inducibility of nitrate reductase activity (NRA) by NO_3^- availability, but short-term darkness did not markedly influence moss NO_3^- uptake or reduction. Significant reduction and isotopic enrichment were detected in moss NO_3^- reserves during N deprivation, showing $^{15}\varepsilon$ of 12.1‰ and $^{18}\varepsilon$ of 14.4‰. The $\Delta\delta^{15}N:\Delta\delta^{18}O$ ratios of ~1 : 1 implied that NRA is the single process driving ^{15}N and ^{18}O fractionations. These results provide new isotopic insights into the nitrate reductase dynamics of the moss.

Additional keywords: denitrifier method, nitrate reduction, nitrate uptake, nitrogen deposition.

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Introduction

The direct utilisation of N from atmospheric deposition is an important N source for terrestrial mosses (Aerts *et al*. 1992; Armitage *et al*. 2012). However, excessive N deposition can engender changes in moss productivity and diversity (Bobbink *et al*. 2010). The capacity of moss layers to retain N deposition was found to be influential on soil N availability for woody plants (Gundale *et al*. 2011). In inorganic N deposition, NO_3^- is generally lower than that of NH_4^+ (e.g. 1–6 and 1–14 kg-N ha^{-1} $year^{-1}$ in European wetlands, respectively, with NH_4-N : NO_3-N ratios between 1 and 2.5) (Bragazza *et al*. 2005), but NO_3^- plays a key role in regulating moss growth and ecophysiological processes (Paulissen *et al*. 2004). Moreover, the NH_4-N : NO_3-N ratio in deposition has been estimated as being likely to decrease in coming decades because of increasing atmospheric NO_3-N (Stevens *et al*. 2011).

Compared with the rooting uptake system of tracheophytes, the rhizoids of mosses serve mainly for anchorage and not for nutrient uptake (Mäkipää 1995). Their substrates usually have little or no NO_3^- availability (Wania *et al*. 2002). Therefore, the process by which mosses acquire (retain and uptake) and use (assimilate) NO_3^- is important for understanding the response and adaptation mechanisms of mosses to increasing NO_3^- deposition (Koranda *et al*. 2007; Salemaa *et al*. 2008; Wanek and Zotz 2011). Moss NO_3^- acquisition is characterised by the fact that NO_3^- can enter moss (typically through cotransport with bound cations) over entire tissue surfaces because of the lack of a cuticular barrier and a lack of stomatal regulation (Raven *et al*. 1998; Glime 2007). For this reason, many mosses were found to absorb N effectively from precipitation solutions (Bayley *et al*. 1987; Marion *et al*. 1987). For instance, Li and Vitt (1997) found that *Sphagnum* moss retained nearly all added NH_4^+-N at the level of 30 kg-N ha^{-1} $year^{-1}$. However, very little evidence exists to help characterise moss NO_3^- uptake and processing mechanisms (Soares and Pearson 1997; Wanek and Pörtl 2008).

The processing of NO_3^- in plants depends mainly on nitrate reductase (NR), which is a substrate-inducible enzyme (Tischner 2000). Therefore, nitrate concentrations ([NO_3^-]) in precipitation is a key factor affecting moss NO_3^- utilisation (e.g. Press and Lee

1982; Woodin *et al*. 1985; Deising and Rudolph 1987). Past analyses of nitrate reductase activity (NRA), mostly performed experimentally on *Sphagnum* species from bog or tundra ecosystems, have revealed that moss NO_3^- utilisation can be induced or inhibited by external [NO_3^-] (Woodin and Lee 1987; Gordon *et al*. 2001; Pearce *et al*. 2003). The regulation of NO_3^- availability by NR in mosses is still uncertain and knowledge should be derived from more species that are conspicuous in broad terrestrial habitats (Koranda *et al*. 2007; Salemaa *et al*. 2008). Aside from NO_3^- availability, light is an important factor influencing NO_3^- uptake and reduction in plants (Gebauer *et al*. 1984; Delhon *et al*. 1995; Peuke and Jeschke 1998), but the light effect has only been tested on a few aquatic mosses (e.g. *Fontinalis antipyretica* L. *ex*. Hedw.; Schwoerbel and Tillmanns 1974).

Methodologically, NRA assay (except for 'the real *in vivo* NRA'; Stewart *et al*. 1993) requires NO_3^- to be added to the substrate medium with plant fragments. The added amount is often much larger than both normal NO_3^- availability and the endogenous NO_3^- pool of natural plants. First, plant physiology and enzymatic dynamics can be changed greatly by the NO_3^- addition, as well as by pH adjustment, vacuum infiltration and other influences. Second, NRA was based on measuring the consumption of the added NO_3^- or the production of nitrite. The influence of high dissolved organic carbon in plant pigments on the colorimetric determination of NO_3^- or nitrite can easily destroy the precision of NRA analysis (especially for 'the real *in vivo* NRA assay' because of very low [NO_3^-]). Third, it is difficult to establish a standard or reasonable NRA assay protocol for diverse plant species. Even for the same plant, using different solution volumes and substrate [NO_3^-] can engender different NR dynamics and NRA results. Therefore, NRA analysis is disadvantageous in terms of the actual reduction ability of NO_3^- in target plants. The application of ^{15}N tracer is a straightforward means to assess the total incorporation of NO_3^- at the level of natural mosses, but it cannot provide information related to the behaviour of NO_3^- after entering moss tissues (Ayres *et al*. 2006; Wanek and Pörtl 2008). Therefore, we used a more sensitive method with the denitrifier without N_2O reductase to convert NO_3^- to N_2O (Casciotti *et al*. 2002) to measure the concentration and stable isotopes ($\delta^{15}N$ and $\delta^{18}O$) of *in vivo* NO_3^- in natural plants (see the detailed merits of this method reviewed in Liu *et al*. 2012*a*, 2012*b*).

Deciphering [NO_3^-] and isotopic signals in mosses entails two important considerations. First, the amount of moss NO_3^- accumulation after receiving precipitation can reflect the uptake in response to NO_3^- availability. Second, dual N and O isotopes of moss NO_3^- directly shed light on the behaviour of NO_3^- (regulated by NRA) after entering moss tissues (Liu *et al*. 2012*a*, 2012*b*). According to the isotopic pattern of the main NO_3^- compartments in plants generalised by Robinson *et al*. (1998) and Comstock (2001), no substantial isotope effect was assumed on the transportation or diffusion of NO_3^- in aqueous solutions. Consequently, tissue NO_3^- isotopes are expected to be similar to the absorbed NO_3^- if it remains unassimilated. An external NO_3^- supply can induce NRA to consume both the tiny original NO_3^- pool and the newly absorbed NO_3^- in plant tissues. Therefore, if the excretion of tissue NO_3^- does not occur, then the residual NO_3^- pool in mosses is often a result of uptake minus reduction, which is expected to be enriched in heavier isotopes (^{15}N and ^{18}O) relative to the source NO_3^- (Mariotti *et al*. 1982; Yoneyama and Kaneko 1989; Evans *et al*. 1996). Recently, greater isotopic enrichment was observed in the NO_3^- of vascular plants at higher NO_3^- availability than those with lower [NO_3^-] and less experience of reduction activity (Liu *et al*. 2012*b*). However, it has not been ascertained whether or not isotopic records of moss NO_3^- in the same moss species would be influenced by the availability of external NO_3^- (Liu *et al*. 2012*a*), or whether or not moss NO_3^- reduction would produce a significant isotopic effect, as seen in fertilised plants ($^{15}\varepsilon = 15‰$; Ledgard *et al*. 1985; Tcherkez and Farquhar 2006). To date, the O isotope effects of NO_3^- reduction have been studied in marine phytoplankton (e.g. Granger *et al*. 2004, 2010). The covariance of $\delta^{15}N : \delta^{18}O$ ratios demonstrated that N–O bond breakage during the reduction of NO_3^- by NR is the single process driving ^{15}N and ^{18}O enrichments in substrate NO_3^-. Similarly, the analysis of tissue NO_3^- isotopes using the denitrifier method enables us to examine the isotopic behaviours of N and O in moss NO_3^-, and thereby ascertain the mechanisms of moss NO_3^- utilisation.

Nevertheless, it is difficult to design hydroponic methods of growing of moss seedlings to simulate the uptake of atmospheric NO_3^- into intact moss layers or to monitor *in vivo* reduction precisely. Field mosses must be washed to remove surface NO_3^- before N treatment and before tissue NO_3^- analysis. Therefore, in this study, the decreasing level of [NO_3^-] in solutions supplied to clean moss tissues was monitored to characterise the short time course and the rate of moss NO_3^- uptake. After short-term incubation of mosses with different NO_3^- solutions under light or dark conditions, the [NO_3^-] and stable isotopes ($\delta^{15}N$ and $\delta^{18}O$) of NO_3^- in moss tissues were measured to interpret the induction or inhibition of NO_3^- reduction by each treatment. Separately, tissue [NO_3^-] and isotopes ($\delta^{15}N$ and $\delta^{18}O$) of the same moss species were checked periodically throughout a 65-day deprivation of N supply to assess their inherent NO_3^- reduction capacity and isotopic effects.

Methods and materials

Moss materials

A pleurocarpous moss (*Hypnum plumaeforme* Wils.) was selected for this study. This species has a worldwide distribution and a wide substrate range including materials such as rotten wood, tree trunks and bases, rock, soil, grassland, sand and clay. The moss was grown at a gardening company (Moss Garden, http://www.mossfarm.jp; Fig. S1, available as Supplementary Material to this paper). Intact moss layers (including new-growing, old and decayed tissues) were moved to the laboratory. The light source was mainly that coming through glass windows. The moss layer had a thickness of ~6 cm and a litter layer of 1–2 cm, but the virtual length of moss individuals was 6–10 cm because of its weft-building feature.

The N content of the moss was 0.9% for green tissues and 0.6% for old tissues ($n = 9$). Moss tissues remained green for ~65 days in the laboratory, even with no N feeding. Deionised water and N-free macronutrient solution (Alghamdi 2003) were supplied to the moss every 2 days until most tissues became brown. Green tissues were collected randomly at 0 ($n=9$), 20 ($n=3$), 49 ($n=6$) and 65 ($n=10$) days from the matrix of moss layers for measuring tissue [NO_3^-] and stable isotopes ($\delta^{15}N$ and $\delta^{18}O$). From each sample, decayed and dead tissues (dark) were discarded after harvest as a whole. Then new (green) and old (brown) tissues were separated immediately according to their distinctive colours. All mosses were washed with deionised water to remove adsorbed impurities and pollutants thoroughly. Washed samples were weighed and dried at 55°C in an oven to constant weight. Then they were finely ground to powder consistency using a ball mill (MM200; Retsch GmbH and Co. KG, Haan, Germany).

Experimental setup and analyses

New moss tissues were washed and filled into clean glass tubes, which were then connected with a reflux solution device through a peristaltic pump (Tokyo Rikakikai, SMP-23, Tokyo, Japan; Fig. S2). The flow rate was controlled at 0.86 mL min^{-1}, with velocity of the liquid flow in the moss column at 0.93 cm min^{-1}. In the first experiment, 47-mL solutions of NO_3^- (as KNO_3, $\delta^{15}N = 1.6 \pm 0.2$‰ and $\delta^{18}O = 26.0 \pm 0.6$‰; $n=10$) in 0, 0.43, 1.73 or 3.48 mg N L^{-1} were fed to new moss tissues (0.81 ± 0.01 g, DW). The NO_3^- of 0.43–3.48 mg N L^{-1} corresponds to wet NO_3^- deposition of 4.3–34.8 kg N ha^{-1} $year^{-1}$ under an annual precipitation of 1000 mm, but our experiments could only mimic [NO_3^-] in rain events. Two 0.5-mL samples were withdrawn every 30 min (i.e. once in each cycle) from each supplied solution to monitor the [NO_3^-] using a NO_x autoanalyser (TRAACS 800; Bran + Luebbe, Tokyo, Japan). Subsequently, the mosses were dried immediately and then ground using the method described above. The water content of the moss was recorded for calibrating the [NO_3^-] in supplied solutions and moss tissues. The NO_3^- withdrawn from solutions for measurements has been considered in calculating the uptake rates.

Because of the finding of extremely efficient NO_3^- removal by moss in the experiment described above, we enhanced the concentration and amount of supplied NO_3^- and decreased the amount of moss specimens in the second experiment to obtain a longer observation time. To old and new tissues (0.58 ± 0.01 g, DW), 75.6-mL solutions of NO_3^- at 0 or 3.90 mg N L^{-1} were given. New tissues were incubated under light and dark (wrapped with aluminium foil) conditions. Every 20 min (each cycle), two 0.5-mL samples were taken from each supplied solution to measure [NO_3^-]. Data in this study are the averages of replicates.

Regarding moss NO_3^- extraction, measurements of tissue [NO_3^-] and stable isotopes ($\delta^{15}N$ and $\delta^{18}O$) followed the same procedures as those introduced in the details presented by Liu *et al.* (2012*a*). Following the eighth *SI Brochure* (Coplen 2011), the extraneous factor of 1000 in the traditional delta definition was avoided and the natural abundances of ^{15}N and ^{18}O ($\delta^{15}N$ and $\delta^{18}O$) were expressed in parts per thousand (per mile) by multiplying them by 1000:

$$\delta^{15}N \text{ or } \delta^{18}O = (R_{sample}/R_{standard}) - 1, \quad (1)$$

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^-.

Isotopic enrichments of NO_3^- in supplied solutions (relative to the initial NO_3^- or plant bulk N, because of the efflux of unassimilated NO_3^-) have been used to interpret the isotopic effects of the NO_3^- utilisation processes (e.g. Evans *et al.* 1996; Granger *et al.* 2004, 2010). The efflux of NO_3^- from moss tissues seems unlikely in our experiments and the complete diminishment of NO_3^- in supplied solutions resulted from moss assimilation. Therefore, the extent of the isotopic enrichment in the residual NO_3^- of moss tissues was used to interpret the inducibility of NRA by external NO_3^- availability. Stable isotopes of endogenous NO_3^- were monitored to examine the isotopic effects imparted by non-induced NRA. The isotopic fractionations caused by NO_3^- reduction were calculated by fitting N and O isotopic ratios of tissue NO_3^- to the Rayleigh equation (Mariotti *et al.* 1981) as:

$$^{15}\varepsilon = (\delta^{15}N_{observed} - \delta^{15}N_{initial})(\ln([NO_3^-]))^{-1} \text{ and} \quad (2)$$

$$^{18}\varepsilon = (\delta^{18}O_{observed} - \delta^{18}O_{initial})(\ln([NO_3^-]))^{-1}, \quad (3)$$

where $^{15}\varepsilon$ and $^{18}\varepsilon$ represent the N and O isotope fractionations respectively.

Calculation of kinetic constants and NO_3^- uptake efficiency

The consumption time and the supplied [NO_3^-] varied within and among treatments (Fig. 1*a, b*). Therefore, the saturation kinetic was not reflected when plotting instantaneous substrate [NO_3^-] and uptake rates (Fig. S3). When a uniform time (90 min) was considered in the first treatment (0.43–3.48 mg N L^{-1}), the uptake of NO_3^- followed a saturation kinetic trend (Fig. S4). Consequently, kinetic constants of NO_3^- uptake were estimated by fitting the uptake data to the Michaelis–Menten equation (regression analysis by hyperbola, single rectangular, two parameters; using SigmaPlot software, ver. 10.0, SPSS Inc., Chicago, IL, USA) as:

$$v = (V_{max} \times [S])/(K_m + [S]), \quad (4)$$

where v is the uptake rate at each substrate [NO_3^-], V_{max} stands for the maximum uptake rate at substrate saturation, K_m is the Michaelis–Menten constant (the substrate [NO_3^-] at which the half-maximal uptake rate is reached), and [S] signifies the substrate [NO_3^-] (Leskovac 2003).

The major purpose of our experiment was to estimate the uptake efficiency of the same moss under different NO_3^- availability and light conditions. V_{max} was derived from the observed maximum uptake rate in each treatment (Table 1). For direct determination of K_m, we chose the approach of the Hanes–Woolf linear transformation:

$$[S]/v = (K_m/V_{max}) + ([S]/V_{max}). \quad (5)$$

The advantage and constraint of this transformation and its application have been discussed and performed concretely by Wanek and Pörtl (2008) with different moss species. Briefly, this method is regarded as producing small only a error in K_m. The uptake efficiency (UE) was calculated as the $V_{max} : K_m$ ratio. The UE enables a comparison of the efficiency of specimens of the same species to take up a specific N form under given

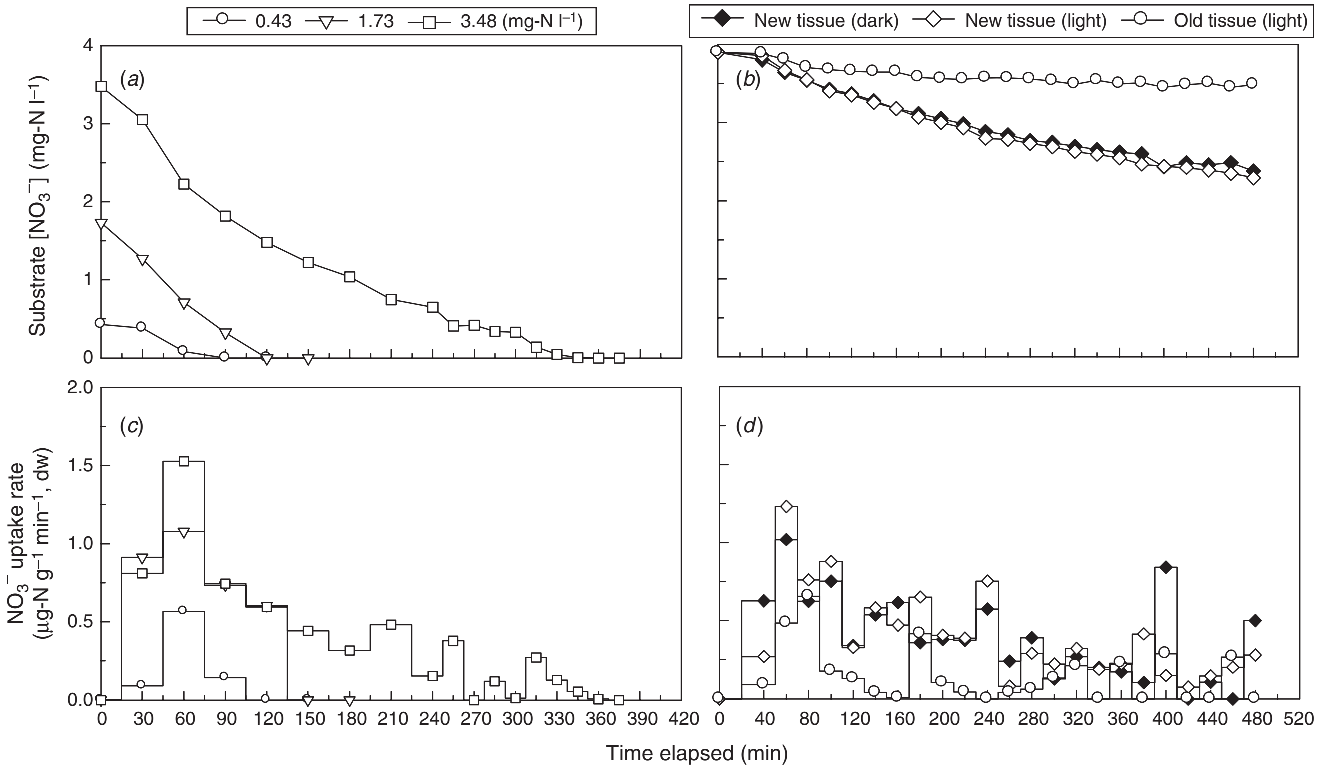

Fig. 1. Time courses of decreasing [NO_3^-] in solutions supplied to the moss *H. plumaeforme* and corresponding uptake rates of NO_3^-. (*a*, *c*) New tissues with different NO_3^- supply; (*b*, *d*) different tissues with the same NO_3^- supply. (*a*, *b*) Substrate [NO_3^-] (mg N L^{-1}); (*c*, *d*) NO_3^- uptake rate (µg N g^{-1} min^{-1}, DW). Data are the averages of two measurements.

Table 1. Kinetic constants of NO_3^- uptake in *H. plumaeforme* tissues after being treated with different [NO_3^-]$_{substrate}$ (mg N L^{-1})

The value of K_m is given in units of mg N L^{-1}, V_{max} in µg N g^{-1} min^{-1} (DW). The uptake efficiency (UE) is the ratio of V_{max} to K_m

Tissue	Light condition	[NO_3^-]$_{substrate}$	K_m	V_{max}	UE
New	Light	0.43	1.09	0.56	0.52
New	Light	1.73	1.63	1.08	0.66
New	Light	3.48	11.48	1.53	0.13
New	Light	3.90	8.46	1.23	0.15
New	Dark	3.90	6.51	1.02	0.16
Old	Light	3.90	14.28	0.66	0.05

environmental and developmental conditions. It potentially reflects the effects of the growth stage (e.g. new and old tissues) and the chemical and physical environment (e.g. light or dark). Therefore, it is suitable for the purposes of estimation in our study.

Statistics

One-way ANOVA was performed to assess the differences in [NO_3^-], δ^{15}N-NO_3^- and δ^{18}O-NO_3^- in the mosses across stages (0 days, 20 days, 49 days and 65 days) of N deprivation. Values are means ± s.d. Single correlation analysis was used to examine the relations among variables. Statistically significant difference was inferred for $P < 0.05$. Statistical analyses were conducted using software (SPSS ver. 13.0 for Windows; SPSS Inc.).

Results

Uptake rate and efficiency

The time for the moss to trap all NO_3^- in uniform solution volume increased with the substrate [NO_3^-]. It was 3–6 times longer in the case of 3.48 mg N L^{-1} than for 0.43–1.73 mg N L^{-1} (Fig. 1*a*). The maximum uptake rate (0.56–1.53 µg N g^{-1} min^{-1}, DW) always occurred at 60 min for new tissues, irrespective of the supplied [NO_3^-] (Fig. 1*c*). The higher the [NO_3^-] in the supplied solution was, the higher the maximum uptake rate (Fig. 1*c*). When initially uniform NO_3^- (3.90 mg N L^{-1}) was supplied continuously to the moss, new tissues showed significantly higher uptake rates than old moss did (Fig. 1*d*). The average uptake rate did not differ between the light and dark treatments (0.38 µg N g^{-1} min^{-1}, DW), although the maximum uptake rate was found to be slightly higher for the moss in the light (Fig. 1*d*). Furthermore, new tissues exhibited the maximum uptake rate at 60 min when supplied with NO_3^- at 3.90 mg N L^{-1}, whereas the maximum uptake rate in old tissues occurred at 90 min (Fig. 1*d*).

In general, the uptake rate was correlated positively with the substrate [NO_3^-] in each treatment with new tissues (Fig. S3). The old tissue showed marked high substrate affinity (K_m value) and low V_{max}, and thereby apparently low UE (0.05) (Table 1). The

K_m increased with V_{max} for new tissues (Fig. S4). The V_{max} was higher for new tissues at the substrate [NO_3^-] of 3.48 mg N L^{-1} and 3.90 mg N L^{-1}, although higher UE (0.52−0.66) was not observed under these high NO_3^- supplies, but was observed under low substrate [NO_3^-] (0.43 mg N L^{-1} and 1.73 mg N L^{-1}) as shown by the low K_m values (Table 1).

Tissue [NO_3^-] and stable isotopes

After incubation with NO_3^- solutions of 0.43−3.48 mg N L^{-1}, new tissues showed lower [NO_3^-] than the control incubated with the NO_3^--free solution, with the lowest concentration (0.57 µg N g^{-1}, DW) at the highest substrate [NO_3^-] (Fig. 2*a*). When supplied with a NO_3^- solution of 3.90 mg N L^{-1} (Fig. 2*b*), the moss showed no substantial NO_3^- accumulation in new tissues or in old tissues compared with its original [NO_3^-]. Calculation (Table S1) revealed that large fractions of the absorbed NO_3^- were assimilated in moss tissues. The NO_3^- originally existing in moss tissues is a minor fraction compared with the absorbed NO_3^- (Table S1) and was estimated to have been consumed quickly by the induced NRA upon the supply of NO_3^-. The isotopic mass balance calculation also revealed that isotopic compositions of the source NO_3^- ($\delta^{15}N = 1.6‰$ and $\delta^{18}O = 26.0‰$, Fig. 3*c–f*) can be regarded as initial isotopic values of the reduced NO_3^- in moss tissues, which implies that the residual NO_3^- pool was derived completely from the supplied NO_3^- solution. The isotopic effects of induced NO_3^- reduction can be based on the isotopic enrichment in residual NO_3^- ($\delta_{residual} - \delta_{absorbed}$) in moss tissues (Fig. 2*c–f*). Furthermore, both the $\delta^{15}N$ and $\delta^{18}O$ of tissue NO_3^- were

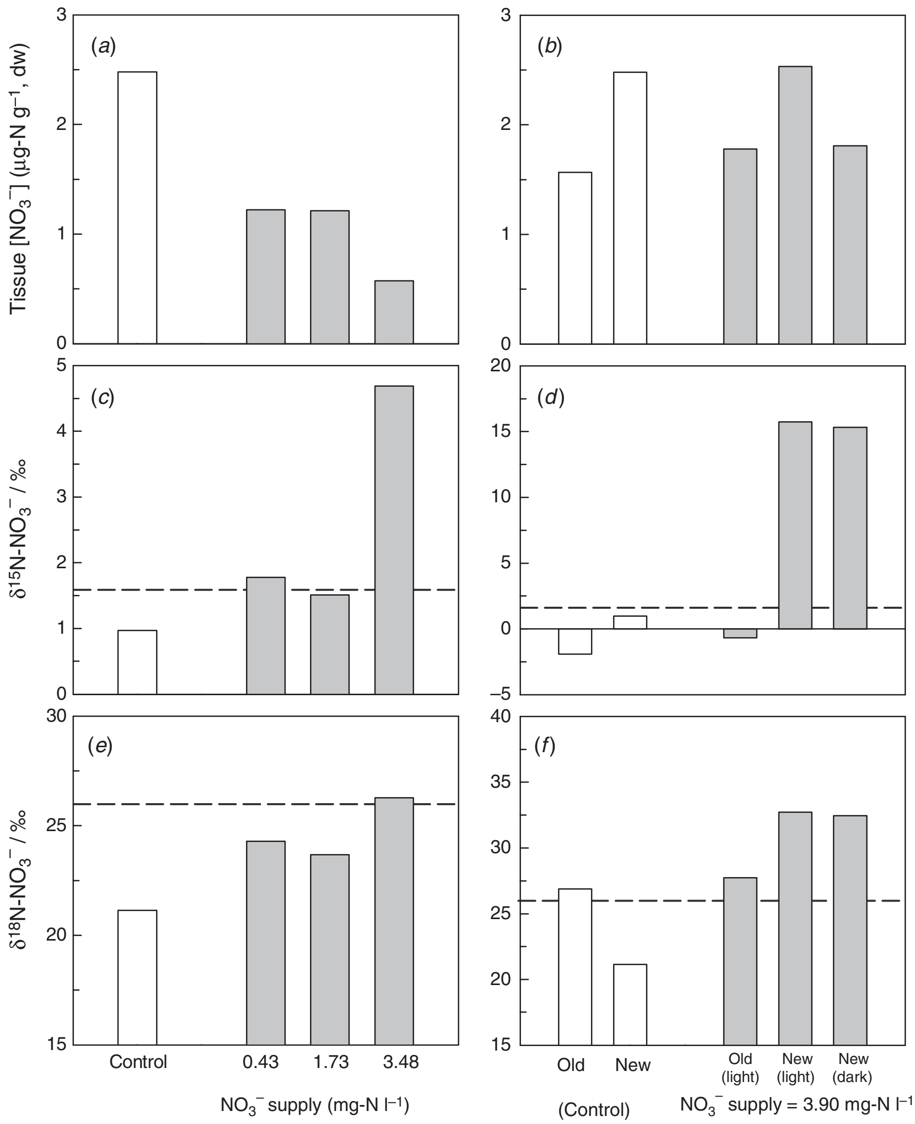

Fig. 2. (*a*, *b*) Concentrations, (*c*, *d*) $\delta^{15}N$ and (*e*, *f*) $\delta^{18}O$ of NO_3^- in moss tissues before (control) and after being fed with NO_3^- in (*a*, *c*, *e*) different concentrations and (*b*, *d*, *f*) light conditions. Dashed lines mark the $\delta^{15}N$ (1.6‰) and $\delta^{18}O$ (26‰) of supplied NO_3^-.

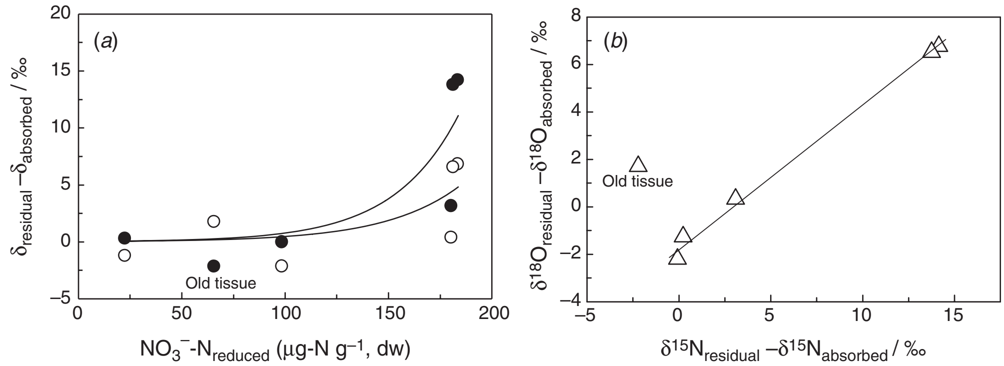

Fig. 3. (*a*) Exponential growth of $\delta_{residual} - \delta_{absorbed}$ with the $[NO_3^-]_{reduced}$ in moss tissues, where (●) represents $\delta^{15}N$ ($y = 0.03e^{0.032x}$, $R^2 = 0.71$, $P < 0.0001$) and (○) represents $\delta^{18}O$ ($y = 0.03e^{0.028x}$, $R^2 = 0.52$, $P < 0.0001$). (*b*) Correlation between $\delta^{15}N_{residual} - \delta^{15}N_{absorbed}$ and $\delta^{18}O_{residual} - \delta^{18}O_{absorbed}$ in *H. plumaeforme* after NO_3^- treatments ($y = 0.47x - 0.31$, $R^2 = 0.80$, $P = 0.02$ for all tissues). The regression line shows the relation for new tissues only ($y = 0.60x - 1.72$, $R^2 = 0.996$, $P < 0.0001$). $[NO_3^-]_{reduced} = [NO_3^-]_{absorbed} - [NO_3^-]_{residual}$, where the $[NO_3^-]_{absorbed}$ (given in µg N g^{-1}, DW) was calculated by monitoring $[NO_3^-]$ in supplied solutions. The $\delta_{absorbed}$ represents isotopic values of the supplied NO_3^-.

enriched even when the NO_3^- was supplied in the dark condition, whereas isotopic compositions of NO_3^- in fertilised old moss did not differ substantially from the control moss (Fig. 2*c–f*). Generally, both $\delta^{15}N$ and $\delta^{18}O$ signatures increased with the NO_3^- reduction (Fig. 3*a*). A strong correlation was found between $\delta^{15}N$ and $\delta^{18}O$ enrichment, but the enrichment of $\delta^{18}O$ in tissue NO_3^- was generally lower than that of $\delta^{15}N$, yielding a linear regression slope of close to 0.6 (Fig. 3*b*).

Throughout the deprivation of N supply, tissue $[NO_3^-]$ decreased to 60% (1.91 ± 0.49 µg N g^{-1}) at 20 days, 50% (1.65 ± 0.42 µg N g^{-1}) at 49 days and 30% (1.09 ± 0.05 µg N g^{-1}) at 65 days relative to the initial level (3.34 ± 0.14 µg N g^{-1}) (Fig. 4*a*). Both the $\delta^{15}N$ and $\delta^{18}O$ signatures of moss NO_3^- increased significantly with a reduction in NO_3^- (Fig. 4*b, c*). A negative correlation was found between the logarithm of tissue $[NO_3^-]$ and isotopic compositions, showing slopes of 14.4‰ for $\delta^{18}O$ and 12.1‰ for $\delta^{15}N$ (Fig. 5*a*). Furthermore, a strong correlation was found between the $\delta^{15}N$ and $\delta^{18}O$ of tissue NO_3^- (slope = 1.12) (Fig. 5*b*).

Discussion

Moss NO_3^- uptake

The temporal patterns of moss NO_3^- uptake (Fig. 1*a*) demonstrated that *H. plumaeforme* is a strong scavenger of accessible NO_3^-. New tissues were able to trap 20.3 µg N in 90 min from the 0.43 mg N L^{-1} NO_3^- solution (at the liquid flow of 0.93 cm min^{-1}). Such efficient uptake of NO_3^- from dilute solutions reflected the potential of natural mosses to survive in low-N habitats (Rudolph and Voigt 1986; Marion *et al*. 1987). Elevated NO_3^- supply extended the uptake time, but the moss still trapped all the supplied NO_3^- in several hours (Fig. 1*a*). Similarly, Bayley *et al*. (1987) found that 99% of NO_3^- (0.3–1.0 mg N L^{-1}) added to a boreal fen was retained by the dominant *Sphagnum* species within 24 h. Li and Vitt (1997) reported that *Sphagnum* was able to trap 50–90% of the deposited N in the field. Mosses are well known to be efficient at absorbing water (95–2225% of their dry mass, Proctor *et al*. 2007; Glime 2007), which facilitates a high uptake efficiency of

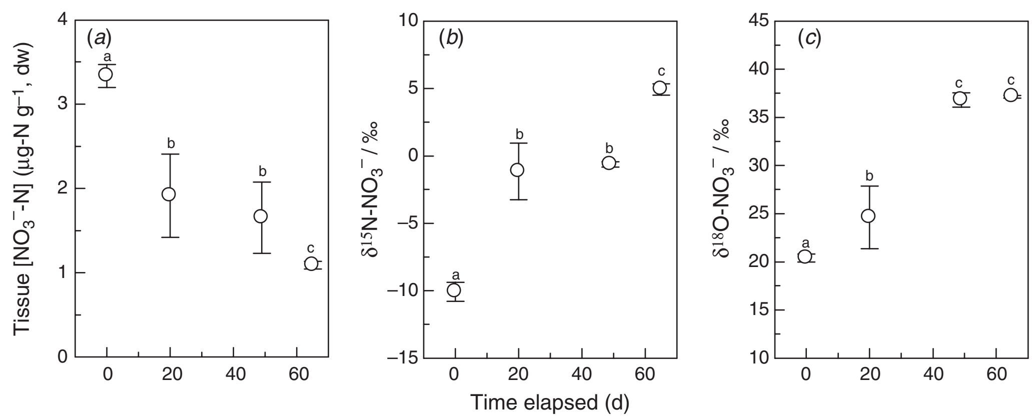

Fig. 4. (*a*) Concentration, (*b*) $\delta^{15}N$ and (*c*) $\delta^{18}O$ of NO_3^- in new growth of the moss *H. plumaeforme* without N supply for 0 days ($n = 9$), 20 days ($n = 3$), 49 days ($n = 6$) and 65 days ($n = 10$). Values (means ± s.d.) not sharing the same superscript letter are significantly different at $P < 0.05$.

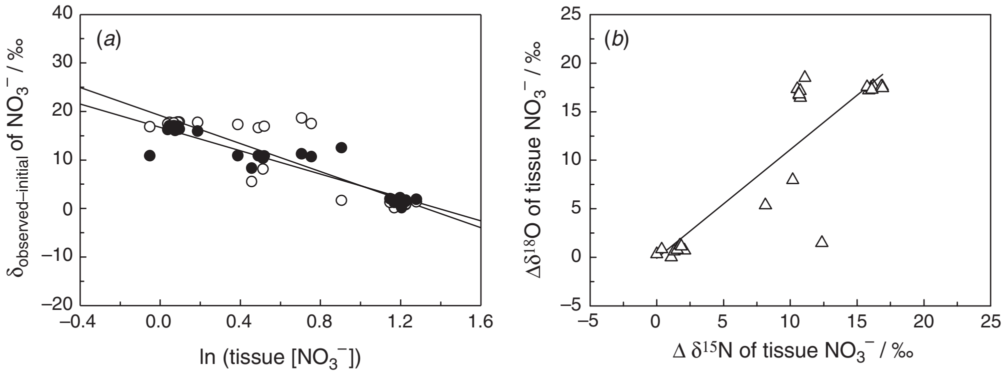

Fig. 5. Correlation between (*a*) ln (tissue [NO_3^-]) and isotopic enrichments of NO_3^- ($\Delta = \delta_{observed} - \delta_{initial}$), where (●) represents δ^{15}N ($y = -12.1x + 16.7$, $R^2 = 0.88$, $P < 0.0001$) and (○) represents δ^{18}O ($y = -14.4x + 19.2$, $R^2 = 0.80$, $P < 0.0001$); and (*b*) between $\Delta\delta^{15}$N and $\Delta\delta^{18}$O (slope = 1.12, $R^2 = 0.80$, $P < 0.0001$) of NO_3^- in *H. plumaeforme* without N supply for 65 days.

N from rain and fog solutions. Our results implied that the *H. plumaeforme*, which is often dominant on forest floors, can potentially attenuate direct NO_3^- inputs to underlying soils (Gundale *et al*. 2011).

Moss NO_3^- uptake occurred upon exposure to NO_3^- (Fig. 1). Deising and Rudolph (1987) also found that NO_3^- uptake in *Sphagnum* showed no time lag. In our experiments, moss NO_3^- uptake is divisible into two phases. First, the uptake rate increased with time and reached the maximum in 60 min (Fig. 1*c, d*), which suggests that the time for *H. plumaeforme* to maximise nitrate uptake rate is constant, irrespective of the applied [NO_3^-] (Fig. 1*b*). After reaching the maximum, the uptake rate decreased with the decrease of substrate [NO_3^-] (Fig. S3), reflecting the regulation of moss NO_3^- uptake rate by NO_3^- availability (Tischner 2000). The increase of V_{max} with the substrate affinity (K_m) of NO_3^- (Fig. S4) also supported the assumption presented above. Previously, the increase of NO_3^- uptake with substrate [NO_3^-] has been observed in algae, fungi, and tracheophytes when NO_3^- was the sole N source (e.g. Gebauer *et al*. 1987; Tischner 2000). The net NO_3^- uptake of hydroponically grown barley (*Hordeum vulgare* L.) can be induced by substrate NO_3^- in both light and dark conditions (Peuke and Jeschke 1998). In our experiment, the mean UE also increased with initial [NO_3^-] when the same supply time (90 min) was considered (Fig. S4). At the later stage (e.g. in 150 min) of incubation with 3.48 mg N L^{-1} of NO_3^-, the moss showed much lower uptake rates than the corresponding substrate [NO_3^-] incubated with 1.73 mg N L^{-1} (Fig. 1*a, c*), suggesting that moss NO_3^- uptake became saturated after reaching V_{max}.

Old mosses were also able to trap NO_3^- (Table 1, Fig. 1*b*). This phenomenon is normal because the revival records of old moss grown in a herbarium were found to be from 3 months to 19 years before water and nutrients were available (Glime 2007; and references therein). Even senescent mosses can maintain a high efficiency of N uptake until major tissue decay commences (Bowden 1991; Bates 2000), suggesting that the senescence does not imply a complete loss of NO_3^- use capacity. The difference was that old tissues exhibited lower UE and uptake rates than new tissues (Table 1). A delay of 30 min was found for old tissues to reach a peak in the uptake rate (Fig. 1*d*). This suggests that NO_3^- consumption by the moss was a function of physiological N demand.

Moss NO_3^- uptake was not influenced substantially by the dark treatment of 8 h, with both light and dark treatments showing comparable UE and V_{max} (Table 1). Similarly, Deising and Rudolph (1987) found that light did not affect NO_3^- uptake in *Sphagnum*. In contrast, some evidence was found to suggest that darkness inhibits NO_3^- uptake because of the limited energy supply (Glass *et al*. 1992), and slower N transport and metabolic processes in plants (Delhon *et al*. 1995; Peuke and Jeschke 1998). Aslam *et al*. (1979) reported that NO_3^- uptake in barley was 20% faster in the light than in the dark. A 30–50% decrease of net NO_3^- uptake rate was observed for soybean (*Glycine max* (L.) Merr.) at 120–360 min after the lights were turned off (Delhon *et al*. 1995). Schwoerbel and Tillmanns (1974) observed that the aquatic moss *Fontinalis antipyretica* var. *gigantea* (Sull.) Sull. could not take up NO_3^- in the dark. Accordingly, the effects of darkness on moss NO_3^- uptake remain uncertain.

Induced NO_3^- reduction and isotopic effects

Excessive NO_3^- supply often causes NO_3^- accumulation in plants (Tischner 2000). In contrast, decreased moss [NO_3^-] was found after short-term NO_3^- incubation, with the lowest level (0.57 μg N g^{-1}, DW) under the highest substrate [NO_3^-] (Fig. 2*a*). These results suggest that moss NO_3^- reduction can be induced by external NO_3^-, which is attributable to the increased rate of reductant supply and metabolism (Tischner 2000). Similarly, Gebauer *et al*. (1984) reported the highest NRA ws found in the in leaf laminae that contained the lowest [NO_3^-], whereas higher [NO_3^-] was found in roots and petioles where NRA was low. Deising and Rudolph (1987) observed NO_3^--triggered NRA and the maximal NRA in *Sphagnum* occurring after 6–8 h of NO_3^- application. In addition to external [NO_3^-], two other possible reasons exist for explaining moss NO_3^- assimilation in our experiments. First, our mosses had at a low or starved N status because of their growth under an N-free condition, and high N demand engenders high UE and assimilation of the resupplied NO_3^-. Waser *et al*. (1999)

reported that diatoms, a common type of phytoplankton, showed lower isotopic fractionations in N assimilation after 48 h of N starvation because of greater N demand and drastically reduced N efflux from the cells. Second, the supply of NO_3^- as KNO_3 might lessen the accumulation of NO_3^- because K^+ can enhance plant NO_3^- assimilation (Blevins *et al*. 1978; Ruiz and Romero 2002).

NRA has been recognised as the major process generating isotopic fractionations in plant NO_3^- assimilation. The major fractionating step was the breakage of the N–O bond by NR (Mariotti *et al*. 1982; Evans *et al*. 1996; Evans 2001). NR is a substrate-inducible enzyme, and isotopic enrichment of residual NO_3^- in the same organs or plants would be therefore greater under the higher NO_3^- supply caused by larger fractions of NO_3^- reduction (relative to the uptake) (Evans 2001; Liu *et al*. 2012*b*). The principle is actually similar to the isotopic enrichment observed in the supplied NO_3^- (relative to the initial NO_3^- or plant bulk N, because of the efflux of unassimilated NO_3^-) (e.g. Evans *et al*. 1996; Granger *et al*. 2004, 2010), because both originate from intracellular reduction. The mechanism described above explains the greater isotopic enrichment of NO_3^- ($\Delta = \delta_{residual} - \delta_{initial}$) in *H. plumaeforme* with higher NO_3^- reduction (Fig. 3*a*). Granger *et al*. (2004, 2010) first reported N and O isotopic effects during NO_3^- assimilation by cultures of phytoplankton strains. Before that, the isotope effects of NO_3^- reduction and assimilation had only been reported for $\delta^{15}N$ in plankton (e.g. Needoba *et al*. 2003; Needoba and Harrison 2004 and cited references) and fertilised vegetables (Ledgard *et al*. 1985; Evans *et al*. 1996). Evidence is lacking in natural plants, especially for $\delta^{18}O$ (Olleros-Izard 1983; Tcherkez and Farquhar 2006). After incubation with NO_3^- solutions of 3.48 mg N L^{-1} or 3.90 mg N L^{-1}, moss NO_3^- showed substantial enrichments in ^{15}N (3.1–14.1‰) and ^{18}O (0.3–6.8‰) (Fig. 2). The range of ^{15}N enrichment was similar to that observed in substrate NO_3^- (11.1‰ and 12.9‰) relative to bulk $\delta^{15}N$ of leaves and roots, respectively (Evans *et al*. 1996). Enrichment in ^{18}O was generally lower than in $\delta^{15}N$, with a $\Delta\delta^{18}O:\Delta\delta^{15}N$ ratio of 0.5 for all tissues and 0.6 for new tissues (Fig. 3*b*). These ratios are similar to the theoretical N and O isotope effects calculated for the dissociation of a single O atom from NO_3^-, which predicts that NO_3^- isotopes will be fractionated in a O : N ratio of ~0.6 (Granger 2006). However, experimental investigations revealed a 1 : 1 trend for $\Delta\delta^{18}O:\Delta\delta^{15}N$ and showed that the enzymatic isotope effects that are intrinsic to NR can be expected to have the same O : N isotopic imprint (Granger *et al*. 2004, 2010). A possible reason for this is that the O exchange between water (the $\delta^{18}O$ of H_2O is ~–8‰ in our experiments) and tissue NO_3^- or NO_2^- during the reduction might cause lower ^{18}O enrichment than that of ^{15}N in tissue NO_3^- (Buchwald and Casciotti 2010).

The lower isotopic enrichment of NO_3^- in older tissues compared to newer tissues (Fig. 2*d, f*) suggests that moss with greater maturity has lower N demand and NRA (Paulissen *et al*. 2004; Liu *et al*. 2012*b*). Isotopic enrichment of NO_3^- in dark-treated mosses resembled that in the light (Fig. 2*d, f*), indicating no substantial influence of darkness on NRA in the moss. Needoba and Harrison (2004) observed higher ^{15}N enrichment in marine diatom species even under no or low light irradiance compared to those with saturating light, showing that dark NO_3^- assimilation did occur in a manner that increases $\delta^{15}N$ fractionation.

Endogenous NO_3^- reduction and isotopic effects

Unlike NO_3^- reduction induced by NO_3^- addition, exogenous NO_3^- deprivation was often used to examine inherent (noninduced) NO_3^- reduction and the activity of endogenous NR (MacKown 1987). Theoretically, internal or vacuolar NO_3^- would be exhausted after the removal of NO_3^- supply for a few hours or days (Gebauer *et al*. 1984; van der Leij *et al*. 1998; Glass *et al*. 2002). However, although the initial level of moss [NO_3^-] (3.34 ± 0.14 µg N g^{-1}, DW) in this study was as low as that of natural mosses (Liu *et al*. 2012*a*), a slow decrease of tissue [NO_3^-] (by 40% in 20 days and 67% in 65 days) was observed following the 65-day period of N deprivation (Fig. 4). Such low and subtle changes of tissue NO_3^- reflected weak NRA in the moss after N deprivation, which were undetectable using traditional methods. Similarly, MacKown (1987) observed that the accumulated NO_3^- in corn (*Zea mays* L.) seedlings decreased apparently after 32 h of NO_3^- deprivation, but a small amount of endogenous NO_3^- remained thereafter because of extremely slow reduction. Two major mechanisms can explain why mosses were unable to use up the endogenous NO_3^-. First, the NR level is controlled mainly by the availability of $NO_3^-$and associated NR-specific proteases; further formation of NR is difficult after N deprivation (Oaks *et al*. 1972; Wallace 1974). Consequently, plants can inherently maintain the balance between NR synthesis and degradation following the removal of exogenous N (Zielke and Filner 1971). Furthermore, part of the residual NO_3^- in new tissues can be translocated from old tissues, which might constitute a strategy that enables younger segments to maintain a basic NO_3^- level after N deprivation. Such intraplant N supply has been found in *Sphagnum* species (e.g. Aerts 1996; Aldous 2002) and *Hylocomium splendens* (Hedw.) B.S.G. (Eckstein and Karlsson 1999).

The isotopic fractionation of NRA has not been evaluated based on isotopic ratios of *in vivo* NO_3^- in natural plants (Tcherkez and Farquhar 2006). Noninduced NO_3^- reduction in the moss *H. plumaeforme* produced isotopic fractionations of 12.1‰ for $\delta^{15}N$ and 14.4‰ for $\delta^{18}O$ (Fig. 5*a*). The $^{15}\varepsilon$ value was similar to that of 15‰ reported on spinach (*Spinacia oleracea* L.) by Ledgard *et al*. (1985), but was generally higher than those observed for marine phytoplankton in natural conditions (4–9‰) and laboratory studies (2.2–6.2‰ (Needoba *et al*. 2003 and cited references) and 0.4–8.6‰, (Granger *et al*. 2010)). The discrepancy might arise from differences in species and growing conditions (e.g. Mariotti *et al*. 1982; Evans *et al*. 1996; Pennock *et al*. 1996). For example, interspecific and intraspecific variations of $^{15}\varepsilon$ and $^{18}\varepsilon$ (5–21‰) were also observed in the NO_3^- assimilation of eukaryotic algae under various conditions (Granger *et al*. 2004). In our experiments, isotope enrichments of moss NO_3^- differed between NO_3^- addition and NO_3^- deprivation, which clearly reflected different NO_3^- assimilation kinetics. The $^{18}\varepsilon$ value was closer to that of 15‰ reported on wheat (*Triticum aestivum* L.) by Olleros-Izard (1983), but higher than those (0.9–8.1‰) reported for strains of prokaryotic plankton

(Granger *et al*. 2010). The covariance of $\Delta\delta^{15}N : \Delta\delta^{18}O$ ratios conformed to a ~1 : 1 trend (Fig. 5*b*), which has been observed in phytoplankton strains (Granger *et al*. 2004, 2010). As processes other than N–O bond breakage (e.g. O exchange; Buchwald and Casciotti 2010) would bias the $\Delta\delta^{18}O : \Delta\delta^{15}N$ line from 1 : 1, our result implied that NRA was the single process causing ^{15}N and ^{18}O enrichments in residual NO_3^-.

Conclusions

Although moss NO_3^- utilisation in response to NO_3^- deposition scenarios is complicated in field settings, this experimental work elucidated key mechanisms in moss NO_3^- uptake and reduction. NO_3^- availability is a major factor regulating the NO_3^- uptake rates in *H. plumaeforme*. Darkness did not affect NO_3^- utilisation during short-term NO_3^- supply. The efficiency of moss NO_3^- uptake was high especially under low $[NO_3^-]$, demonstrating two phases during the consumption of accessible NO_3^- in a given precipitation event. Initially, the NO_3^- uptake rate increased and maximised within a constant time. Subsequently, the uptake efficiency decreased, especially for precipitation with high $[NO_3^-]$ and long duration. Larger isotopic enrichment of moss NO_3^- occurred under higher NO_3^- availability and NO_3^- uptake, reflecting the inducibility of NRA by external NO_3^-. The NO_3^- reserves in *H. plumaeforme* were detectable even when the N supply had been excluded for 65 days, during which time, isotopes of tissue NO_3^- increased with the reduction of NO_3^-. Coupled N and O isotopic enrichment (~1 : 1) revealed that the NRA was the sole fractionating step in moss NO_3^- utilisation after N deprivation.

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