Tracing uptake and assimilation of NO$_2$ in spruce needles with $^{13}$N

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Received 24 April 1995; Accepted 27 July 1995

Abstract

For the first time, spruce shoots (Picea abies [L.] Karst.) were fumigated in vivo with $^{13}$N-labelled NO$_2$ with the aim of elucidating the mechanism of NO$_2$-trapping in the apoplast of the substomatal cavity. Uptake by the needles could be monitored on-line, and a quantitative analysis of the activity records delivered a deposition velocity in agreement with the common dry deposition estimates and ruled out rapid export processes. A fast extraction procedure was applied which revealed that NO$_2$ did not produce any detectable traces of nitrite. In needles in which the enzymes of nitrate reduction were not induced by prior fumigation with NO$_2$, incorporation of NO$_2$ was partially inhibited as compared to the fully induced shoots which took up and assimilated NO$_2$ as expected from a constant influx. The only labelled inorganic species found in the extracts was nitrate (60%), whereas the rest of the label (40%) was assimilated organic nitrogen. A quantitative analysis of the data shows that the reaction of NO$_2$ in the apoplast yields at least three times more nitrate than nitrite, so that the existing models about the apoplastic trapping reaction, disproportionation or antioxidant scavenging, which both postulate substantial production of nitrite, have to be reconsidered.

Key words: $^{13}$N, nitrogen dioxide, spruce, air pollutants, deposition.

Introduction

Dry deposition of reduced or oxidized nitrogen compounds via the common gas exchange routes may represent a significant contribution to the nutrition of plants in nitrogen-limited ecosystems (Skeffington and Wilson, 1988; Bender et al., 1990; Duyzer and Fowler, 1994). Increased nitrogen loads may play an important role in forest decline and affect biodiversity in the sensitive ecosystem types (Schulze, 1989; Wellburn, 1990; Nåsholm et al., 1994). The parametrizations used in models for estimates of deposition of nitrogen dioxide should rely on quantitative knowledge about the processes involved at the plant level. Stomatal control of NO$_2$-uptake in plant leaves, including coniferous forest species, is widely accepted (Thoene et al., 1991; Duyzer and Fowler, 1994), although there is some discussion about possible internal resistances to incorporation at low NO$_2$ concentrations below 2 ppb. NO$_2$-N enters nitrogen metabolism and is incorporated into amino acids and proteins (Nussbaum et al., 1993), and, especially in coniferous forests, its role as a fertilizer contrasts with possible adverse effects such as nutrient imbalances (Wellburn, 1990) or changed shoot:root mass ratios.

Atmospheric NO$_2$ at ecological levels induces nitrate reductase (NR), the rate-limiting enzyme in nitrate assimilation, in shoots of Norway spruce (Picea abies [L.] Karst.) and other conifers (Wingsle et al., 1987; Norby et al., 1989; Thoene et al., 1991; von Ballmoos et al., 1993), which tend to assimilate nitrate or ammonium in the roots and thus show low constitutive levels of NR activity in shoots. A linear relationship between external NO$_2$ concentration, and NR activity has been detected (von Ballmoos et al., 1993). The rate of uptake of NO$_2$ depended linearly on stomatal conductance in a wide range of NO$_2$-concentrations. Experiments using non-radioactive $^{15}$N have shown that NO$_2$-N is fully integrated...
in the assimilatory pathway and incorporated into amino acids (Nussbaum et al., 1993). This requires transformation of gaseous NO \(_2\) to a dissolved, anionic form in the apoplast. The measured uptake rates could not be explained by aqueous disproportionation of NO \(_2\) to nitrate and nitrite with the kinetic parameters given by Lee and Schwartz (1981). As an alternative mechanism, scavenging of NO \(_2\) by an apoplastic antioxidant (ascorbate) was proposed on the basis of high ascorbate concentrations found in apoplastic liquids (Ramage et al., 1993), but has not yet been verified experimentally to our knowledge. A discrepancy remained between the induction of NR by NO \(_2\) and this radical H-abstraction reaction yielding nitrite only, as the availability of nitrate is regarded as the main factor controlling the activity of this enzyme (Rajasekhar and Oelmüller, 1987), and a rapid oxidation of nitrite in the cytoplasm seems unlikely.

The short-lived radioactive tracer \(^{13}\text{N}\) (half-life 9.96 min) is a powerful tool for the investigation of nitrate transport (Meeks, 1993; Presland and McNaughton, 1986; Siddiqi et al., 1991). Up to now, only a preliminary fumigation of barley using \(^{13}\text{NO}_2\) has been reported (Rowland, 1985). A gas chemical technique providing \(^{13}\text{NO}_2\) has recently been established at the accelerator facility of the Paul Scherrer Institute, Switzerland (Baltensperger et al., 1993). Detection of \(\gamma\)-rays emitted after positron decay of \(^{13}\text{N}\) allows non-destructive and \textit{in situ} monitoring of \(^{13}\text{NO}_2\) uptake as well as sensitive detection of \(^{13}\text{N}\) in fractions of plant extracts. Here we present results from the fumigation of spruce shoots with \(^{13}\text{N}\)-labelled \(^{13}\text{NO}_2\), including an extraction procedure to determine the biochemical speciation of \(^{13}\text{N}\) after uptake. The short life time requires rather fast and straightforward experimental procedures.

Materials and methods
Preparation of \(^{13}\text{NO}_2\)
\(^{13}\text{N}\) was produced according to the reaction \(^{16}\text{O}(p,e)^{13}\text{N}\) with a gas target (2% \(\text{O}_2\) in He) and a proton beam (14 MeV, 1.5 \(\mu\)A) of the Philips Cyclotron at the Paul Scherrer Institute, Villigen, Switzerland. The verification of \(^{13}\text{N}\) as predominant radioactive product with a half-life of 596 s as well as its gas chemical speciation under the present experimental conditions has been described by Baltensperger et al. (1993). The continuous flow gas chemistry set-up yielded \(^{13}\text{NO}_2\) at a flow rate of 16.7 cm\(^3\) s\(^{-1}\), which was transported through a 1 km long polyethylene capillary of 4 mm diameter to the laboratory for exposure of the trees (transport time c. 10 min resulting in an activity loss of about 50% due to decay). After steady-state had been established, no \(^{13}\text{NO}_2\) losses to the tube walls were observed. The pressure drop between the target chamber and the exposure set-up at ambient pressure was about 0.6 bar. The flux of \(^{13}\text{NO}\) to the exposure set-up was continuously monitored by measuring \(\gamma\)-activity in the capillary wound around a NaI-detector. First, all \(^{13}\text{NO}\) was converted to \(^{13}\text{NO}_2\) by passing the gas over a solid oxidizer, a 7 cm long tube filled with firebrick sucked with equal weights of \(\text{CrO}_3\), \(\text{H}_2\text{O}\), and \(\text{H}_3\text{PO}_4\) (Levaggi et al., 1972). Efficient conversion was checked periodically by passing the gas through a trap of firebrick impregnated with triethanol amine (for trapping NO\(_2\)) followed by a trap of \(\text{Co}_3\text{O}_4\) on quartz chips (for trapping NO\(_2\)). Relative activity in the second trap never exceeded 5% of the first. The gas was then mixed with the same volume of water-saturated ambient air to which carrier NO\(_2\) from a certified bottle containing 2000 ppm NO\(_2\) in N\(_2\) (Carba Gas, Switzerland) was added with a calibrated mass flow controller. The final composition of the fumigation gas was 81 ppb NO\(_2\) labelled with \(10^6\) \(^{13}\text{NO}_2\) cm\(^{-3}\) and 44% He in ambient air at a total flow rate of 1970 cm\(^3\) s\(^{-1}\), with a relative humidity of 50% at 20°C (all gas volumes are standard volumes throughout the paper).

Plant material and pretreatment
Fifty 2-year-old spruce trees (\textit{Picea abies} [L.] Karst.) grown in the nursery from seeds of the same site were potted at the end of March in polyethylene pots (51) containing 40% bark compost, 40% peat and 20% sand. The pots were placed in climate controlled growth chambers at 200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR in daily light periods increasing from 10 h to 16 h from April to June. Relative humidity was about 75% at 20°C throughout the period. The air supplied was preconditioned with a particle filter and a permanganate oxidizing absorption filter to remove the nitrogen oxides. The growth of the shoots was completed by the end of May. After transfer of the trees to the Paul Scherrer Institute, 10 trees of similar size were placed 10 d before the start of the experiments in two identical fumigation chambers (40 cm x 35 cm x 60 cm) made of PVC and covered with acrylic glass. The fumigation chambers were used for pretreatment of plants with charcoal filtered air (A) or NO\(_2\) (B). In each chamber PAR provided by metal halogen lamps was about 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) for the upper shoots. Day/night cycles were 16/8 h throughout the whole period of pretreatment and the experiments. Air flow supplied by ventilators between charcoal filter and chamber was approximately 0.1 m\(^3\) min\(^{-1}\). In chamber B, NO\(_2\) was added from a cylinder containing 1000 ppm NO\(_2\) in N\(_2\) with a calibrated mass flow controller to give a concentration of 80 ppb. All NO\(_2\) concentrations during growth and pretreatment were checked routinely with a chemiluminescence detector (Model 8841, Monitor Labs, USA). The pretreatment with NO\(_2\) guaranteed induction of nitrate reductase in the needles (von Ballmoos et al., 1993). In control experiments prior to the present study, the final level of activity was reached after 2-3 d and remained constant for at least 6 d.

Fumigation
Before every fumigation a tree from the pretreatment chamber A or B was selected and transferred to a hood in the gas chemistry laboratory and placed below an identical light source. One current year shoot was cautiously enclosed into a glass cuvette of 30 mm inner diameter held horizontally below the lamp. Before every fumigation a tree from the pretreatment chamber A or B was selected and transferred to a hood in the gas chemistry laboratory and placed below an identical light source. One current year shoot was cautiously enclosed into a glass cuvette of 30 mm inner diameter held horizontally below the lamp. Before every fumigation a tree from the pretreatment chamber A or B was selected and transferred to a hood in the gas chemistry laboratory and placed below an identical light source.
enclosing the shoot to monitor on-line the activity from $^{13}$N taken up by the needles.

**Extraction procedure**

The end of each fumigation marked the zero time point for all corrections of activity measurements. The fumigation cuvette was opened immediately, the shoot cut off from the tree and dipped in 12 ml CHCl$_3$ for 10 s to remove most of the soluble wax covering the surface and stomata (Günthardt-Goerg, 1986), and all $^{13}$N adhering to it. The dry shoot was submersed in liquid nitrogen to separate the needles from the twig. The needles (200-400 mg fresh weight) were homogenized with a Polytron (Kinematica, Switzerland) in 4 ml H$_2$O at full speed for 10 s. An aliquot of the homogenate (1 ml) was boiled in a water bath for 5 min to expel nitrite. In control experiments, nitrite added to the acidic homogenate (pH=4.0) at the ppm level was removed quantitatively by this treatment, without affecting nitrate. Both boiled and unboiled homogenates, were centrifuged at 4000 g for 5 min. The supernatant of the unboiled homogenate was filtered (0.2 μm, FP030/3, Schleicher & Schuell, USA) and applied to 0.7 cm x 0.5 cm of a Polygosil C18 column to retain soluble organic material. The eluate was then injected into an anion chromatography system (Hamilton, PRP-100, eluting with 0.7 ml min$^{-1}$ of 4mM potassium-dihydrogenphthalate). Radioactivity was measured in aliquots of the CHCl$_3$ extract, the bare needles, the homogenate prior to and after boiling, the supernatants and pellets of boiled and unboiled homogenates, the remains on the C18-column and its eluate. Measurements were made with an NaI-detector shielded with lead and allowing identical counting efficiency for all measurements. Each sample was counted for at least 20 s. The detection limit was 2 cps. The whole needles had accumulated several hundred cps at the end of fumigation. The eluate leaving the anion exchange column was fed directly into the detector so that an on-line cumulative radiochromatogram was obtained, from which the retention time was determined. All activity measurements were corrected for decay during the time elapsed since the end of fumigation (22 min until detection of anions), for fresh weight of harvested needles, and for label concentration in the fumigation gas. Each fumigation of 5, 10, 20, 30, and 60 min, as well as the subsequent extraction of needles, was repeated with two individual trees for each pretreatment. All fumigations were conducted between 2 and 10 p.m. on three subsequent days.

**Results**

**Analysis of on-line data and deposition velocity**

Uptake of $^{13}$NO$_2$ by the needles could be monitored on-line during every fumigation. With a constant total uptake rate, $f$ (labelled molecules per second), the amount of deposited labelled molecules, $n$, increases linearly with time and decays with the constant, $\lambda$, so that the activity at the shoot, $a$, should follow an exponential saturation curve:

$$\frac{dn}{dt} = -\lambda \cdot n + f = a = f \cdot [1 - \exp(-\lambda \cdot t)]$$ (1)

With a half-life of 9.96 min for $^{13}$N ($\lambda = 0.00116$), saturation completes after about 1 h. Figure 1 depicts the on-line record of a fumigation of a tree from pretreatment B, where, instead of the extraction procedure, the radioactive gas was shut off after about 1 h and decay of the activity in the needles was observed *in situ* under otherwise constant conditions during another hour. From two such fumigations, linear regression on the logarithm of the decaying signal (Fig. 1, insert) resulted in a mean half-life of 9.98 min, indicating that no substantial removal mechanism of $^{13}$N products, such as export to roots or release back to the gas, had to be taken into account within the experimental period. The background activity from the bulk volume of the labelled gas and adsorption of label to the walls of the empty cuvette was below 5% of the measured activity at the shoot.
Stepwise inversion of the activity signal, \( \{a_j, a'\} \), by
\[
f_j = \frac{a_{j+1} - a_j e^{-d_{j+1} - d_j}}{f - e^{-d_{j+1} - d_j}}
\]
and averaging over 10 min, yielded the actual flux, \( f \), of label to the needles as function of time, \( t \) (solid line in Fig. 1), and revealed that the flux of label was approximately constant during the fumigation period. No difference in the shape of the saturation curves was observed for the pretreatments A and B. The reason for this behaviour will be elucidated in the following sections providing information about the distribution of activity within the needles. Within this section, the two pretreatments are not treated separately.

The leaf area-based deposition velocity, \( v_d \), is defined by
\[
v_d = \frac{f}{A \cdot c}
\]
where \( c \) is the concentration of the labelled molecules and \( A \) the total surface area of exposed needles. \( f \) is obtained from the saturation activity, \( a_p \), of the curves such as in Fig. 1, whereas \( c \) can be derived from the saturation activity, \( a_o \), observed with the spruce twig replaced by the TEA-impregnated firebrick absorbing all incoming \( ^{13} \text{NO}_2 \) at a flow rate \( \phi \):
\[
f = \epsilon_1 \cdot a_o, \quad c = \epsilon_2 \cdot \frac{a_o}{\phi}.
\]
The problem of quantitative assessment of counting efficiencies, \( \epsilon_1 \) and \( \epsilon_2 \), of the detector is overcome by measuring the activities, \( a_o \) and \( a_p \), with the same geometrical configuration. \( v_d \) then becomes
\[
v_d = \frac{a_o \cdot \phi}{A \cdot c}.
\]
The mean parameters obtained from four fumigations of 60 min duration were \( a_o = 15000 \text{ cps}, \phi = 32.8 \text{ cm}^3 \text{ s}^{-1}, a_p = 625 \text{ cps}, \) and \( A = 14 \text{ cm}^2 \) based on a mean surface to fresh weight ratio of 70 \text{ cm}^2 \text{ g}^{-1}, so that the deposition velocity turned out to be 0.1 ± 0.03 cm s\(^{-1}\).

To test for stomatal control of the observed uptake, one fumigation of 20 min duration was started 15 min after the lights had been turned off. The specific activity in the needles dropped to about one-third of that found for the same fumigation time in the light. The complete offset of stomatal \( \text{NO}_2 \) deposition due to closure of stomata needs about 1 h (Thoene, 1991).

**Analysis of needle extracts**

\( ^{13} \text{N} \) adhering to the needle surface at the end of fumigation is given as fraction of the total deposited label, i.e. the sum of the activity in the \( \text{CHCl}_3 \) extract and the bare needles. With \( \text{NO}_2 \) pretreatment, this fraction was 9 ± 3% compared to 18 ± 8% for trees not exposed to \( \text{NO}_2 \) previously. In the latter case, the surface fraction tended to increase with fumigation time, whereas it was constant in the former.

In the following, all activity not extractable by \( \text{CHCl}_3 \), is denoted as incorporated activity. Overall recovery of activity in the pellets, on the C18-columns, and in its eluate, compared to that found in the bare needles prior to homogenization was 98 ± 13%, independent of pretreatment and fumigation time. As the intact needles did not fill a constant volume on the detector independent of the amount of needles, which led to deviations in the activity measurement, only the sum of activities in the fractions of the extracts was used for quantitative analysis as incorporated activity. Boiling the homogenates did not cause activity loss, as would have been expected for labelled nitrite, but increased the fraction of activity in the pellets by 23 ± 4% compared to the unboiled homogenates. This increase was assigned to soluble proteins precipitated by boiling. In control experiments the amount of proteins remaining dissolved in the cold homogenate was only 1–2% of the total protein content. It was concluded that the extracts did not contain substantial amounts of nitrite and that no label was released from precipitated material to the supernatant such as anions attaching to positively charged membrane lipids (Skokut et al., 1978). Activity in the eluate of the C18-column (presumably inorganic ions) was retained on the anion exchange column as nitrate, and no indication of another negatively or positively charged species (ammonium) was found. Figure 2 presents the specific radioactivity in the extract fractions, now definitively assigned to proteins (pellet), amino acids (column) and nitrate (eluate), as a function of the fumigation time, for both pretreatments, A and B, charcoal-filtered air and 80 ppb \( \text{NO}_2 \), respectively. In case A, the total incorporated activity decreased by 40% between 20 and 60 min, corresponding to a drop in the influx by 50% after 20 min, whereas in needles pretreated with \( \text{NO}_2 \) the shape of the activity increase followed the expected exponential saturation curve (Equation 1) for constant influx. For both pretreatments, the relative distribution among the protein, the amino acid and the nitrate fraction was approximately 18%, 22%, and 60%, respectively, largely independent of fumigation time.

**Discussion**

The on-line activity records showed that the flux of label to the enclosed shoot was approximately constant during the fumigations. Thus, the gas composition (presence of \( \text{He} \) and a lower \( \text{CO}_2 \) concentration due to mixing the reactive gas with ambient air) appeared not to have a substantial influence on gas exchange. A somewhat higher flux in the beginning in some of the fumigations may be
explained by a slight closure of stomata due to increasing temperatures within the cuvette. The presence of He in the fumigation gas certainly increased to some extent the diffusion coefficient of NO$_2$ compared to air, whereas the relative humidity of 50% did not allow the stomata to open completely. With these restrictions, the leaf area-based deposition velocity of 0.1 cm s$^{-1}$ agreed fairly well with published data on NO$_2$ deposition to spruce needles (Nussbaum et al., 1993; Thoene et al., 1991; Hanson and Lindberg, 1991). In view of the off-line extraction experiments, no rapid export mechanism to stem and roots or from the needle surface back to the gas have to be taken into account within the time-scale dealt with.

The results from experiments on the induction of NR in spruce needles by NO$_2$ concentrations between 2 and 60 ppb (von Ballmoos et al., 1993) brought up the question whether the NO$_2$-uptake may be inhibited after an increase in NO$_2$ concentration before NR activity reaches a level high enough to cope with the incoming NO$_2$. The present results showed that the amount of labelled N incorporated into the needles indeed decreased after a short increase, when NR was not induced prior to fumigation. This decrease seemed to coincide with an increased adsorption of NO$_2$ on the waxy needle surface, so that the total flux of NO$_2$ to the needles remained approximately constant during the first hour of fumigation, as observed on-line. No accumulation of nitrate was found in the extracts, so that nothing is known about a possible inhibition mechanism. Closure of stomata after the onset of the NO$_2$ fumigation cannot be completely ruled out, as no direct measurements of gas exchange had been made. Further experiments must decide whether this effect points to an important process occurring on the large surface of epistomal waxes in spruce needles. Therefore, the following discussion is restricted to an analysis of data obtained with trees which were pretreated with 80 ppb NO$_2$ prior to the fumigation (B), where the fumigation with $^{13}$N-labelled NO$_2$ traced a steady-state flux into the needles.

The fact that the distribution among organic and nitrate-N was mostly independent of fumigation time suggested that reduction of nitrate, i.e. the first step in assimilation of NO$_2$-N, was rate-limiting. To estimate the kinetic parameters from the data presented in Fig. 2B, we applied a simple linear model (Fig. 3) of assimilation of $^{13}$NO$_2$-N, which assumed nitrate reduction as the only limiting process (keeping in mind that the constant flux of NO$_2$ into the substomatal cavity is presumably controlled by stomatal opening (Thoene et al., 1991)). $\gamma$, $\beta$, and $\lambda$ denote exchange constants for radioactive decay, reduction of nitrate and of nitrite, respectively. Input to the system is allowed as constant fluxes, $f_1$ and $f_2$, for nitrate and nitrite, respectively. $f_2$ is assumed to contribute directly to the organic fraction (regarding nitrite and ammonium as transient pools with fast turnover compared to radioactive decay). Export from the organic fraction other than radioactive decay was neglected, according to the on-line analysis (Fig. 1).
Fig. 3. Linear model of trapping and assimilation of $^{15}$NO$_2$-N by spruce needles. The model assumes that influx of nitrite and nitrate are constant with time, that nitrate reduction limits nitrate assimilation and that incorporation of nitrite into the organic fraction is faster than radioactive decay. Greek letters denote exchange constants for radioactive decay ($\lambda$), nitrate reduction ($\beta$) and nitrite reduction ($\gamma$), $f_1$ and $f_2$ the influx of nitrate and nitrite, respectively.

This system was described by the following set of equations:

$$\frac{dn_{tot}}{dt} = (f_1 + f_2) - \lambda \cdot n_{org}$$

$$\frac{dn_{nitr}}{dt} = f_1 - (\lambda + \beta) \cdot n_{nitr}$$

$$\frac{dn_{org}}{dt} = f_2 - \lambda \cdot n_{org} + \beta \cdot n_{nitr}$$

(6)

where $n_{tot}$, $n_{nitr}$, and $n_{org}$ denote label concentrations of total incorporated, nitrate and organic nitrogen (sum of amino acids and proteins), respectively. The total flux, $f_1 + f_2$, was estimated by fitting $n_{tot}$ to the experimental data (Fig. 2B), total incorporated activity). From $n_{nitr}$ a value of $\beta$ corresponding to a half-life of about 30 min was obtained. Based on these parameters, the ratio of nitrate to nitrite influx, $f_1/f_2$, was estimated from $n_{org}$ and $n_{nitr}$ to be 3.3. These parameters were used to calculate (Fig. 4) the accumulation of incorporated, non-radioactive carrier NO$_2$-N, i.e. $\lambda = 0$ in Equation 6. Thus, the observed partition of incorporated nitrogen between the nitrate and the organic fraction was the result of a much higher nitrate (>75%) than nitrite influx (<25%), $f_1$ and $f_2$, respectively. A study comparing the export and reduction fluxes from the cytoplasm of root cells (Lee and Clarkson, 1986) suggests slightly lower half-lives for nitrate reduction in the range of 10 to 20 min compared to 30 min suggested by the present analysis. A lower half-life applied to our experimental data would even increase the estimated ratio of nitrate to nitrite influx (3.3), which we therefore regard as a lower limit. Due to the low direct nitrite influx, $f_2$, and fast removal of nitrite by nitrite reductase and glutamine synthetase (Skokut et al., 1978) compared to radioactive decay, the nitrite concentration remained below our detection limit. Macroscopic nitrite accumulation in conifers was observed in an over-winter fumigation (Wolfenden et al., 1991) and was due to inhibition of the reducing enzyme. Another report of nitrite found in plant extracts is given by Zeevart (1976), who fumigated tobacco with exceedingly high NO$_2$ concentrations. Even though the reduction of nitrate by NR appeared to be the rate-limiting step in the assimilation of NO$_2$-N, it is still fast enough to keep the resulting concentration of nitrate in spruce needles at a rather low level (40 nmol g$^{-1}$, Fig. 4).

According to our model and its fit to the present experimental data, any process yielding nitrite directly from NO$_2$, and thus by-passing NR, contributed only to a minor degree to the uptake. Consequently, neither the disproportionation-model according to Lee and Schwartz (1981), predicting identical amounts of NO$_3$ and NO$_2$, nor the apoplastic-antioxidant-hypothesis predicting NO$_2$ only (Ramge et al., 1993), can be considered to explain our data. A recent re-analysis of the heterogeneous trapping of NO$_2$ at aqueous surfaces (Bambauer et al., 1994) showed that, in contrast to nitrate, nitrite may be revolatilized as HONO from an acidic solution. Since for conifers apoplastic pH-values between 5 and 5.5 have been reported (Pfanz and Dietz, 1987), that study may provide a hypothesis for the ratio of nitrate to nitrite reported here. Under conditions similar to those in a substomatal cavity, i.e. with a high liquid surface to gas volume ratio, the trapping reaction rate turned out to be first order in NO$_2$ and not second order as reported previously (Lee and Schwartz, 1981). Combined with our
findings, these results shed a new light on the relation between the aqueous trapping mechanism and the uptake rates in plants at ecological NO$_2$ concentrations.

Future studies should concentrate on pulse-chase experiments to determine the exact half-life for reduction of nitrate. In addition, the $^{13}$N-techniques presented here offer the unique possibility to study the relation between stomatal conductance, uptake and assimilation rates of NO$_2$-N at the very low NO$_2$-concentrations representative of remote forested areas.

Acknowledgements

We thank B. Eichler, and D. Piquet for their contribution to the experimental setup at the Paul Scherrer Institute. We greatly appreciate the support by M.S. Günthardt-Goerg who provided us with the methods of needle wax extraction.

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