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Vertical structure and diurnal variability of ammonia exchange potential within an intensively managed grass canopy

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Abstract

Stomatal ammonia compensation points (χ_s) of grass species on a mixed fertilized grassland were determined by measurements of apoplastic NH_4^+ and H^+ in the field. Calculated χ_s-values were compared with in-canopy atmospheric NH_3 concentrations (χ_a) measured by micrometeorological techniques.

Leaf apoplastic NH_4^+ did not significantly differ between intact leaves from different heights above the ground. Bulk leaf NH_4^+ and especially NO_3^- slightly increased at the bottom of the canopy and these concentrations were very high in senescent plant litter. Calculated χ_s-values were below atmospheric χ_a at all canopy levels measured, indicating that the grassland was characterized by NH_3 deposition before cutting. This was confirmed by the χ_a profile, showing the lowest χ_a close to the ground (15 cm above soil surface) and an increase in χ_a with canopy height, especially during the night. Neither χ_s nor χ_a could be measured close to the soil surface, the litter NH_4^+ material indicated a high potential for NH_3 emission tough.

A diurnal course in apoplastic NH_4^+ was seen in the regrowing grass growing after cutting, with highest concentration around noon. Both apoplastic and tissue NH_4^+ increased in young grass compared to tall grass. Following cutting, in-canopy gradients of atmospheric χ_a showed NH_3 emission but since calculated χ_s-values of the cut grass were still lower than atmospheric NH_3 concentrations, the emissions could not entirely be explained by stomatal NH_3 loss. High tissue NH_4^+ in the senescent plant material indicated that this fraction constituted an NH_3 source. After fertilization, NH_4^+ increased both in apoplast and leaf tissue with the most pronounced increase in former compared to the latter. The diurnal pattern in apoplastic NH_4^+ was even more pronounced after fertilization and calculated χ_s-values were generally higher, but remained below atmospheric NH_3.

1 Introduction

Several investigations have revealed the bidirectional character of NH_3 exchange between vegetation and the atmosphere with large fluctuations on annual, seasonal and daily time scales (Sutton et al., 1995; Bussink et al., 1996; Hermann et al., 2001; Horvath et al., 2005; Walker et al., 2006; Sutton et al., 2007). In a non-fertilized managed grassland in The Netherlands, NH_3 emission fluxes were frequent (about 50% of the time) during a warm and dry summer period, while in a wet and cool autumn period deposition fluxes dominated (80% of the time; Kruit et al., 2007).

The direction of the NH_3 flux between plant leaves and the atmosphere depends on the stomatal NH_3 compensation point (χ_s) of leaves, which is the atmospheric NH_3 concentration where NH_3 emission and deposition are balanced and no net exchange occurs (Farquhar et al., 1980; Husted et al., 1996). In chamber studies χ_s was shown to be influenced by the N status of the plant (Sharpe and Harper, 1995; Mattsson et al., 1998; Mattsson and Schjoerring, 2002; Sommer et al., 2004) and by environmental factors such as temperature (Mattsson et al., 1997), photosynthetic photon flux density and air humidity (Mattsson and Schjoerring, 1996; Husted and Schjoerring, 1996; Husted et al., 2002).

Measurements of vertical NH_3 concentration gradients within a grass/clover canopy (Denmead et al., 1976) and a quackgrass (*Agropyron repens* L.) canopy (Lemon and van Houtte, 1980) showed a sharp increase of the NH_3 concentration towards the soil surface, resulting in a upward NH_3 flux from the soil to the base of the grass canopy. Similarly, a more recent study based on the inverse Lagrangian source/sink analysis for an oilseed rape (*Brassica napus*) canopy also revealed highest NH_3 concentrations at the ground level, which was suggested to originate from decomposing litter leaves (Nemitz et al., 2000). This was supported by a very high ammonium NH_4^+ concentration measured in senescent plant material from oilseed rape compared to the concentration in intact leaves (Husted et al., 2000). It is not known yet, if corresponding NH_4^+ gradients between leaves of different age may occur in perennial grass species.

A diurnal pattern of the NH_3 exchange has been observed in *Brassica napus* (Husted et al., 2000), barley (Schjoerring et al., 1993) and grassland (Trebs et al., 2006), with highest NH_3 emission rates typically during the daytime and low rates at night. Since diurnal variations in apoplastic NH_4^+ and H^+ concentrations seem to be small (Husted et al., 2000; van Hove et al., 2002), changes in NH_3 emission may be attributed to temperature effects on NH_3 solubility and NH_4^+ dissociation in the apoplast due to varying canopy temperature during the diurnal course (Husted and Schjoerring, 1996). In addition, fluctuations in leaf surface wetness will affect the NH_3 exchange (Walker et al., 2006; Kruit et al., 2007). Diurnal variations of NH_3 emission have also been observed over grassland, but correlation between the measured atmospheric χ_a and χ_s, calculated from flux density measurements, was low (Harper et al., 1996).

The experiment presented here was carried out in May and June 2000 in Braunschweig, Germany and was part of a joint investigation within the EU GRAMINAE project (Sutton et al., 2008[1]). The aim was to estimate the NH_3 exchange potential of the vegetation on a vertical gradient within a fertilized grass canopy and its diurnal fluctuations by means of χ_s. The vacuum infiltration technique for apoplast extraction was directly applied in the field and calculated χ_s was related to in-canopy NH_3 concentrations. It is discussed whether leaf tissue NH_4^+ could be a useful indicator of χ_s, since measuring this parameter would be more convenient and less time-consuming than the determination of χ_s.

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2 Materials and methods

2.1 Description and management of the measurement site

The measurement site was located near Braunschweig (52°18′N, 10°26′E , 79 m a.s.l.) in Lower Saxony, Germany. The field was 600×300 m in size and consisted of a mixed sward dominated by *Lolium perenne* L. (see footnote 1). It has been a grassland for 4 years, typically receiving 250 kg N ha^{-1} a^{-1}. Prevailing wind directions were SW to W and E. A farm with 300 cattle and 3000 pigs was located in the W of the field. The field was cut on 29 May and N fertilizer (100 kg N ha^{-1}) was applied as calcium ammonium nitrate on 5 June. Further details of the experimental set up and site conditions are reported by Sutton et al. (see footnote 1).

2.2 Micrometeorological measurements

Instruments for the measurement of χ_a were placed in the centre of the field. χ_a was measured continuously online by Mini Wet Effluent Denuders (mini-WEDD), as described by Neftel et al. (1998), connected to a four-channel fluorescent analyzer. Before cutting three of the Mini-WEDDs were placed within the plant canopy and one directly above the canopy. Air flow rates of 200 ml min^{-1} and 800 ml min^{-1} were used for the lowest two mini-WEDDs and for the two above, respectively. A liquid flow of 0.12 ml min^{-1} was used and the detection limit was 0.1 μg NH_3 m^{-3}.

2.3 Sampling of plant material

During the first period of the experiment, a few days before the field was cut, plant material was collected from different layers within the plant canopy and separated into flowers, stems and leaf sheaths and green and brown leaf laminae. The fully developed green leaf laminae were used for apoplast extraction as described below. After the cut it was no longer possible to properly divide plant material into different species. Therefore a mixture of cut leaves from all the species was collected. The plant material was randomly collected in the field and immediately brought to an adjacent field lab. Some of the leaves were used for extraction directly after sampling and the plant material used for the determination of tissue NH_4^+ and NO_3^- was immediately frozen in liquid nitrogen and stored at −20°C.

2.4 Apoplast extraction

Apoplast liquid was extracted by means of vacuum infiltration (Husted and Schjoerring, 1995) modified as follows: Whole leaf laminae or cut leaves were infiltrated with a 280 mM sorbitol solution at a pressure of 16 bar for 5 s followed by vacuum. This procedure was repeated 5 times. After infiltration, leaves were carefully blotted dry, packed into plastic bags and equilibrated for 20 min in the daylight and after that centrifuged for 10 min at 4°C and 800 g. Concentrations of NH_4^+ in the extracted solution were determined by flow injection analysis (FIA) or HPLC analysis (Waters Corp., Milford, USA) using *o*-phthalaldehyde (OPA) as reagent as described by Genfa and Dasgupta (1989). Apoplastic pH was measured with a Micro-Combination pH electrode (type 9810, Orion, Beverly, USA). In order to assess cytoplasmic contamination of the apoplasts, malate dehydrogenase (E.C. 1.1.1.38) activity was determined and compared with the activity measured in bulk leaf extracts (Husted and Schjoerring, 1995). Cytoplasmic contamination was below 1.5% for all considered plant species.

2.5 Stomatal NH_3 compensation points

Estimates of χ_s in grass species were obtained from measured apoplastic pH and NH_4^+ concentration by the following equation:

$$\chi_s = K_H \cdot K_d \cdot \left(\frac{NH_x}{K_d} + [H^+] \right) \tag{1}$$

where NH_x is the NH_4^+ and NH_3concentration of the apoplast and K_H and K_d the thermodynamic constants of $10^{-9.25}$ and $10^{-1.76}$ at 25°C, respectively. χ_s was adjusted to actual canopy temperature by the Clausius-Clapeyron equation:

$$\ln\left(\frac{\chi_2}{\chi_1}\right) = \frac{\Delta H^0_{\mathrm{dis}} + \Delta H^0_{\mathrm{vap}}}{R \cdot \left(\frac{1}{T_1} - \frac{1}{T_2}\right)} \tag{2}$$

where χ_1 is the NH_3 compensation point at the temperature T_1 and χ_2 the NH_3 compensation point at the actual canopy temperature T_2. Canopy T was measured by an IR Pyranometer. Enthalpies of dissociation of $NH_4^+ o\Delta H^0_{\mathrm{dis}}$) and vaporization of $NH_3(_g\Delta H^0_{\mathrm{vap}})$ are 52.21 kJ mol^{-1} and 34.18 kJ mol^{-1}, respectively, and R denotes the gas constant (8.31 J K^{-1} mol^{-1}).

Γ-values represent a measure of the NH_3 exchange potential independent of temperature and are calculated as follows:

$$\Gamma = \frac{\text{apoplastic } NH_4^+}{\text{apoplastic } H^+} \tag{3}$$

2.6 Determination of bulk tissue $NH_4{}^+$ and ($NO_3{}^-$)

0.2 g of the frozen plant material was homogenized to powder and was extracted in 2 ml 10 mM formic acid in a cooled mortar containing a little quartz sand. The extract was centrifuged at 25 000 g and 4°C for 10 min. The supernatant was transferred to 500 μl 0.45 μm polysulphone centrifugation filters (Micro VectraSpin, Whatman Ltd., Maidstone, UK) and spun at 5000 g and 4°C for 5 min. $NH_4{}^+$ and $NO_3{}^-$ of the supernatant was analyzed using a flow injection system (Quik Chem instrument, Lachat Instruments INC, Milwaukee, USA).

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3 Results

3.1 Vertical structure of NH_3 exchange potential

Before the field was cut plant material was collected from four different layers, in order to characterise the vertical structure of $[NH_4^+]$ and NO_3^- of the plants. The fully developed canopy was 76 cm high at that stage. Green leaf laminae, which were used for apoplast extraction, were found in all the layers except in the top level (60–70 cm) (Fig. 1). Brown senescent leaves constituted an additional fraction in the lowest canopy layer (0–20 cm), but uncontaminated apoplast liquid could not be obtained from this fraction. Apoplastic NH_4^+ was highest in young leaves occurring at the upper layer of the plant (Fig. 2A). Yet the difference between the layers was not significant ($p>0.05$) due to relatively large variability between the replicates, especially in the top canopy layer. Leaf apoplastic pH ranged between 6.3 and 6.6 in all the layers (Fig. 2B). Tissue NH_4^+ was much higher in brown senescing leaves close to the soil surface compared to green leaves at the same canopy height (Fig. 2C). NO_3^- of stems and green leaves decreased with canopy height (Fig. 2D) and was highest in the stems except in the layer closest to the ground where NO_3^- was higher in the leaves. Like apoplastic NH_4^+, χ_s did not differ significantly between the different layers and the values were below the measured in-canopy χ_a (Fig. 3).

3.2 Diurnal course of NH_3 exchange potential

Before the cut, the most abundant plant species *Lolium perenne* and *Phleum pratense* were selected for determination of the NH_3 exchange potential during a diurnal course. The course of apoplastic NH_4^+ as well as Γ (NH_4^+/H^+) in non senescent green leaves as shown for *Lolium perenne* in Fig. 4A and C did not show any particular pattern whereas apoplastic pH was higher during the night than during the day (Fig. 4B). After the field was cut, apoplastic NH_4^+ of grass leaves was generally higher and a distinct diurnal course could be seen on the first day, with highest apoplastic NH_4^+ before

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noon and a decrease during the night (Fig. 4A). However, apoplastic NH_4^+ remained low on the following day probably because of the lower canopy temperature on the second day compared to the day before. However, the increase in NH_4^+ following the cut was more pronounced in the leaf tissue and was also observed on the second day (Fig. 5A). In contrast, NO_3^- seemed to decrease during the day and an increase was observed during the night. (Fig. 5B). Like before the cut, highest apoplastic pH was measured in the night (Fig. 4B). Due to generally lower apoplastic pH of the cut grass mix compared to the grass before cutting Γ was similar before and after the cut (Fig. 4C). After fertilization NH_4^+ increased in both the apoplast and the tissue (Fig. 4A and 5A). The diurnal pattern in apoplastic NH_4^+ and Γ was more pronounced after N application than before. Before fertilization a relatively good correlation was seen between leaf tissue and apoplastic NH_4^+, which was significant $p<0.01$ after cutting but not before cutting (Fig. 6). Because apoplastic NH_4^+ increased while tissue NH_4^+ was rather unaffected after fertilization, the correlation between tissue and apoplastic NH_4^+ was very low.

Before the field was cut the vertical profile of χ_a was predominantly characterised by decreasing χ_a towards the ground as shown for a diurnal course in Fig. 7. This χ_a profile would therefore indicate NH_3 deposition from the atmosphere to the plant canopy. Calculated χ_s of both *Lolium perenne* and *Phleum pratense*, which corresponded to the upper two χ_a measuring heights, were below the in-canopy χ_a. The increase in χ_a during the night was not reflected in χ_s. An inverseχ_a profile was observed after the canopy had been cut. At the lowest measuring height χ_a reached 10 $\mu g\, m^{-3}$ in the morning and χ_a decreased with measuring height (Fig. 8). χ_a was lower during the night than during the day. Accordingly, highest NH_3 emission was measured during the day (Milford et al., 2008[2]). Generally, χ_s of the cut grass were much lower than

[2]Milford, C., Theobald, M. R., Nemitz, E. N., Hargreaves, K. J., Horvath, L., Raso, J., Daemmgen, U., Neftel, A., Jones, S., Hensen, A., Loubet, B., and Sutton, M. A.: Ammonia fluxes in relation to cutting and fertilization of intensively managed grassland derived from an intercomparison of gradient measurements, Biogeosciences, to be submitted.

χ_a above the plant canopy. The same direction of the slope of the vertical χ_a gradient but higher concentrations during the day were seen after N application (Fig. 9). A typical diurnal pattern with highest concentration around noon was most pronounced after fertilization and was reflected in both calculated χ_s and atmospheric χ_a. Although χ_s of the fertilized grass were about five times higher than before fertilization the values were still below atmospheric χ_a of the lowest measuring height during the whole diurnal course.

4 Discussion

Application of the vacuum infiltration technique directly in the field enabled an immediate extraction of apoplast liquid and therefore frequent determination of the NH_3 exchange potential of the plants during a diurnal course. The measured apoplastic ${NH_4}^+$ levels before fertilization were about 0.1 mM (Fig. 4A) matching values reported in pastures under similar N conditions by Herrmann et al. (2001) and Loubet et al. (2002). Considerably higher apoplastic ${NH_4}^+$ concentrations, 0.2 to 0.9 mM, were observed in an intensively managed grassland in The Netherlands throughout the growing season (van Hove et al., 2002). The nitrogen availability in the soil particularly that of ammonium, has a profound influence on apoplastic ${NH_4}^+$ concentrations as also demonstrated by the increase following fertilisation (Fig. 4A) (Mattsson et al., 2008).

Apoplastic ${NH_4}^+$ and χ_s increased by a factor of two from the bottom to the top of the intact plant canopy (Figs. 2A and 3). Thus, young leaves had a relatively high NH_3 emission potential. At all in-canopy levels considered, χ_s was below the measured atmospheric χ_a, indicating that plants acted as NH_3 sinks. This was confirmed by the measured NH_3 flux which was characterized by NH_3 deposition (see footnote Milford et al.) and is in agreement with measurements carried out over a grass/clover canopy (Herrmann et al ., 2001).

The NH_3 emission measured from the field after the cut could not be totally explained by a raise in χ_s of the cut grass. χ_s of the senescent plant material either attached to the stubbles or lying on the ground could, however, not be calculated since apoplastic infiltration of senescent plant material could not be achieved. Yet, very high tissue NH_4^+ measured in plant litter, which accounted for about 20% of the total above ground biomass after the cut, indicate that this fraction may represent an important NH_3 source. This might explain the NH_3 emission measured after cutting, when the litter fraction was not covered by a canopy and no absorption from the intact leaves could occur anymore. Husted et al. (2000) showed that in an oilseed rape field, the plant litter fraction represented an NH_3 source, while attached leaves acted as NH_3 sinks. Similarly, in a grass/clover crop the highest in-canopy χ_a was found towards the soil surface (Denmead et al., 1976). In the present investigation atmospheric NH_3 could not be measured below 15 cm and therefore NH_3 concentration directly above the soil surface is not known. However, using a tissue NH_4^+ value for brown leaves as presented in Fig. 2C and a measured pH of 7 (data not shown) would result in Γ-values for the litter of about 5000. Although this Γ value cannot be considered as a direct measure of the effective NH_3 emission of plant litter it still indicates a high potential for NH_3 emission. Furthermore, NH_3 flux measurements carried out in a climate chamber study revealed a NH_3 emission of about 10 nmol m^{-2} leaf area s^{-1} from cut senescent leaf material *of Lolium perenne* (Mattsson and Schjoerring, 2003). This would result in a NH_3 emission of about 80 ng m^{-2} s^{-1} using the amount of litter biomass per surface area of 20% of total as measured in the present investigation (David et al., 2008[3]).

While plant litter emission could explain the measured NH_3 emission after the cut it cannot entirely account for the high emission observed after fertilization (see footnote 2). Directly after N application most of the NH_3 emission most probably originated from fertilizer particles lying on the ground (Herrmann et al,. 2001). Yet, the NH_3 emission measured over the following days and its distinct diurnal pattern (see footnote 2) indicate that another NH_3 source than fertilizer must be involved. Although χ_s of the grass

[3] David, M., Roche, R., Mattsson, M., Sutton, M. A., Schjoerring, J. K., Daemmgen, U., and Cellier, P.: Analysis of ammonia fluxes with intensively managed grassland using dynamic chambers II: the effect of management options, Biogeosciences, to be submitted.

considerably increasedafter fertilization (Fig. 4C) it still remained below measured atmospheric χ_a and thus plants should represent an NH_3 sink.

A discrepancy between micrometeorological or cuvette studies and the bioassay approach in estimating χ_s has been observed in several investigations. In most of these studies the bioassay approach yielded smaller estimates of χ_s compared to the micrometerological or cuvette measurements (Mattsson et al., 1997, Hill et al., 2001; Mattsson and Schjoerring, 2002). Considering a possible underestimation of χ_s in the present study, NH_3 emission from the plants would become likely, especially after cutting and fertilization around midday, when the ratio between χ_a and estimated χ_s was smaller than during the rest of the day. However, the discrepancy between χ_a and estimated χ_s was still considerable for most of the collected data, indicating that also after fertilization other NH_3 sources might be involved in the NH_3 exchange of the canopy.

The diurnal measurements clearly showed that apoplastic NH_4^+ may change during the course of the day, with highest values around midday and decreasing concentrations during the night. This pattern was also reflected in Γ which is an indicator for the NH_3 exchange potential of a plant g but in contrast to χ_s, it is independent of any change in canopy temperature. This is different from observations made in an oilseed rape field, where no diurnal variation in Γ existed and where canopy temperature was the only factor influencing χ_s on a diurnal scale (Husted et al., 2000).

Before fertilization a relatively clear linear relationship existed between leaf tissue NH_4^+ and apoplastic NH_4^+ (Fig. 6), but this was not the case after fertilization. In addition, the ratio between tissue NH_4^+ and apoplastic (NH_4^+) was much lower after fertilization compared to before fertilization. These findings differ from studies in a Scottish grassland, where the magnitude of increase in NH_4^+ after cutting was similar for the apoplastic and bulk tissue fraction (Loubet et al., 2002). Also in two grass species grown with different N supply the correlation between apoplast and leaf tissue NH_4^+ was fairly good (Mattsson and Schjoerring, 2002) while in a wild perennial the same correlation was poor (Hill et al., 2002). The data presented here indicate that

NH_4^+ in the tissue and in the apoplast may be regulated independently and thus the tissue NH_4^+ can not always be used as an indicator of χ_s.

5 Conclusions

From the present investigation we conclude that the plants of a fully developed grassland acted as NH_3 sinks and that NH_3 was predominantly deposited to the tall canopy. NH_3 emission measured after the cut and after fertilization could not entirely be accounted for by stomatal loss. Yet, elevated tissue NH_4^+ and high Γ-values in especially senescent plant material indicated that NH_3 might be emitted from plant litter, which could explain the NH_3 emission measured after cutting. Although Mattsson et al. (2008) showed a high inter-species correlation between Γ and bulk leaf NH_4^+, this comparison shows that there are limitations in this relationship when considering temporal differences for individual species. Specifically, the relationship was shown to change after fertilization, indicating that bulk tissue NH_4^+ should only be used as an indicator of Γ when calibration specific to current conditions is available.

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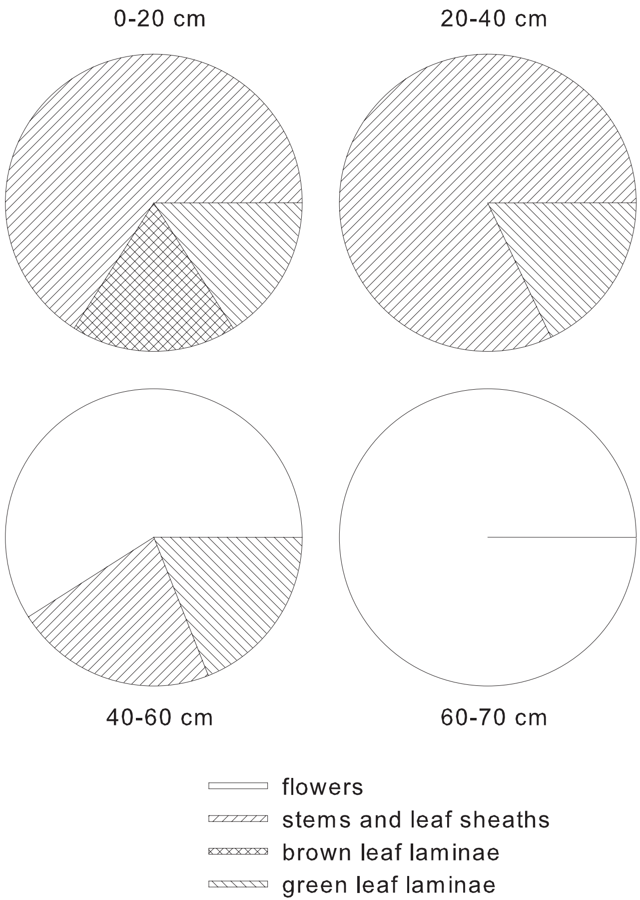

Fig. 1. Relative contribution of the fresh weight of flowers, stems and leaf sheaths, and green and brown leaf laminae to total plant biomass at different layers within the plant canopy.

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Ammonia exchange potential within a grass canopy

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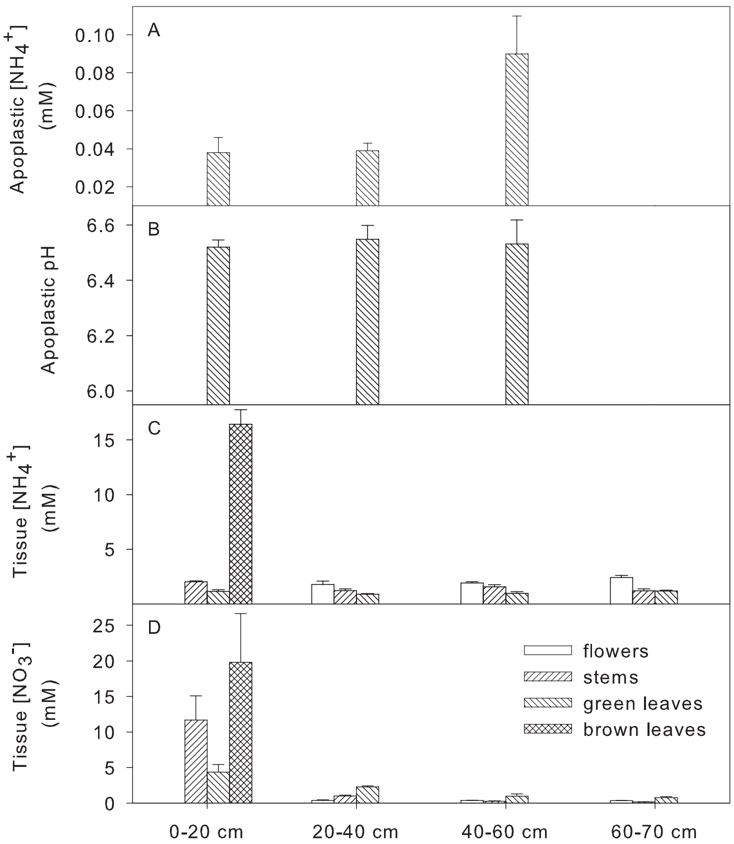

Fig. 2. Apoplastic NH_4^+ **(A)** and pH **(B)**, bulk NH_4^+ **(C)** and NO_3^- **(D)** of grass plants at different heights within the intact canopy on 29 May. For the highest level apoplasticdata are means of the dominant species *Lolium perenne* and *Phleum pratense* weighted for species abundance (n=8±SE) whereas for the other levels a mixture of all species was considered (n=4±SE).

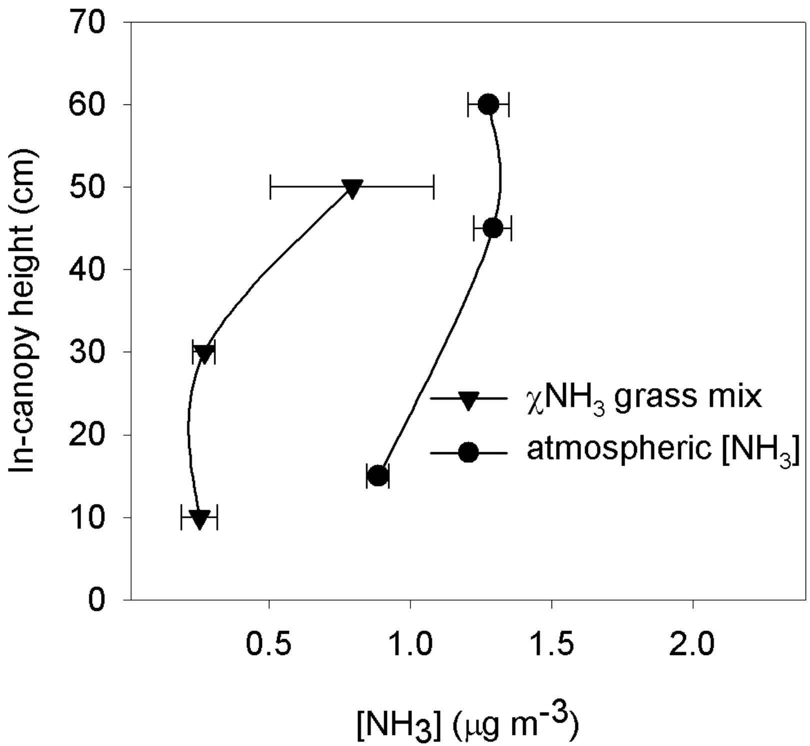

Fig. 3. NH_3 profile within the intact grass canopy and calculated mean χ_{NH_3} of grass leaves on 29 May. For the highest level χ_{NH_3} data are means of the dominant species *Lolium perenne* and *Phleum pratense* weighted for species abundance (n=8±SE) whereas for the other levels a mixture of all species was considered (n=4±SE). NH_3 represent mean concentrations over three days before cutting (10:00 a.m.–16:00 p.m.). The dashed line indicates a potential course of χ_{NH_3} when calculating χ_{NH_3} from tissue water pH and NH_4^+ for the litter fraction.

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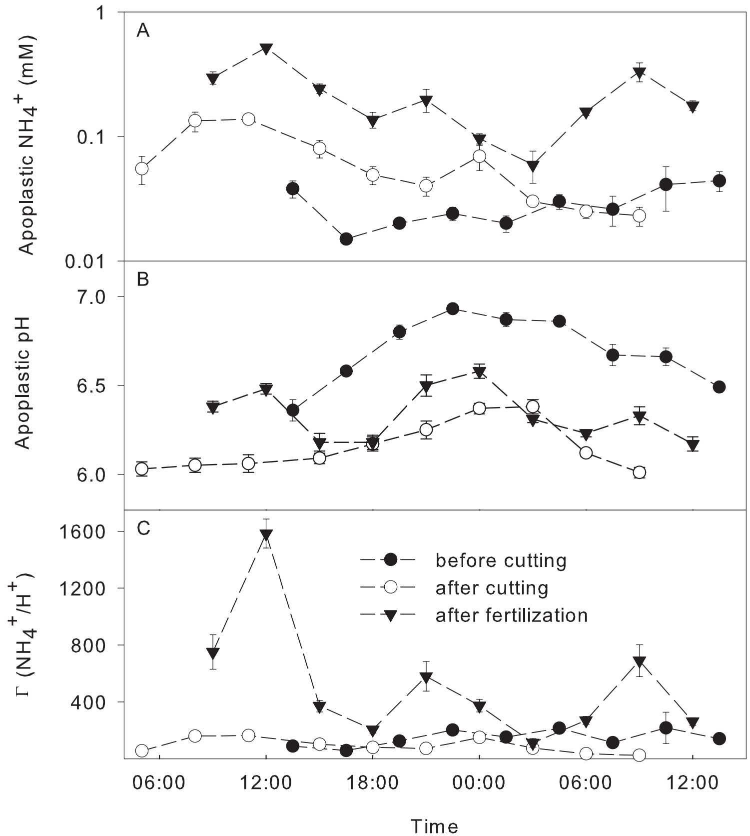

Fig. 4. Diurnal course of apoplastic NH_4^+ **(A)**, apoplastic pH **(B)** and Γ (apoplastic NH_4^+/H^+) **(C)** in grass leaves before and after cutting and after fertilization. Data are means of 4 replicates±SE and represent a mixture of all species except before fertilization when data represent the most dominant species *Lolium perenne*.

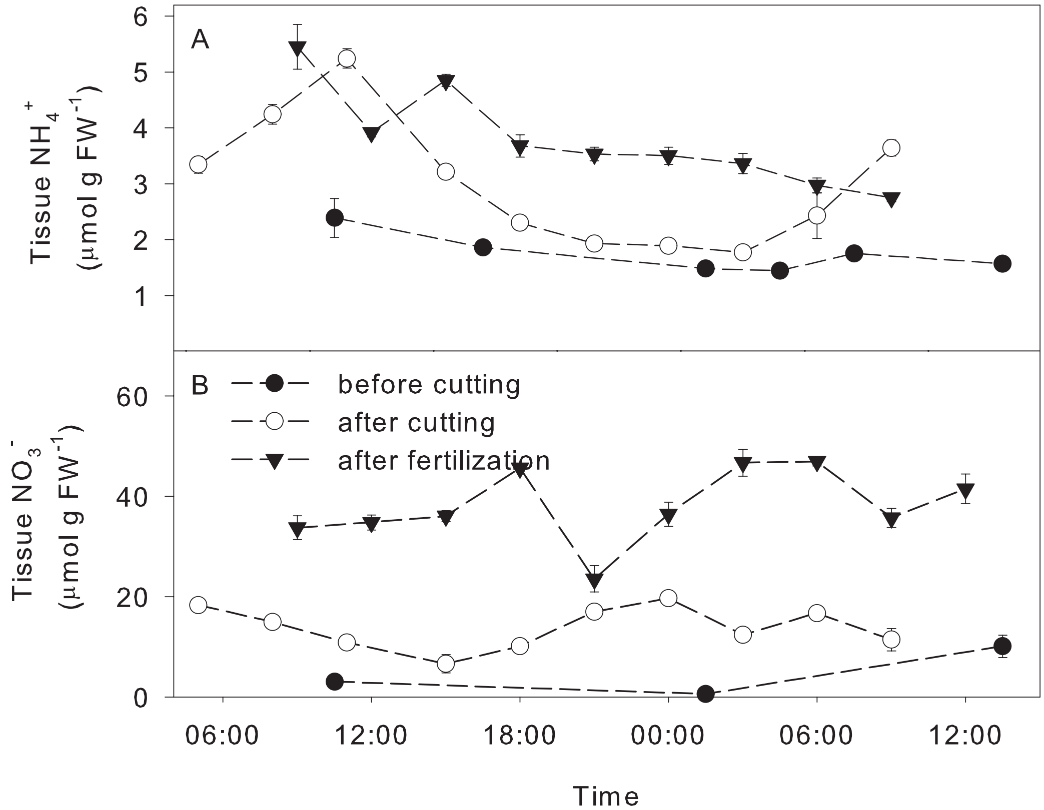

Fig. 5. Diurnal course of bulk NH_4^+ **(A)** and NO_3^- **(B)** in grass leaves before and after cutting and after fertilization. Data are means of 4 replicates±SE and represent a mixture of all species.

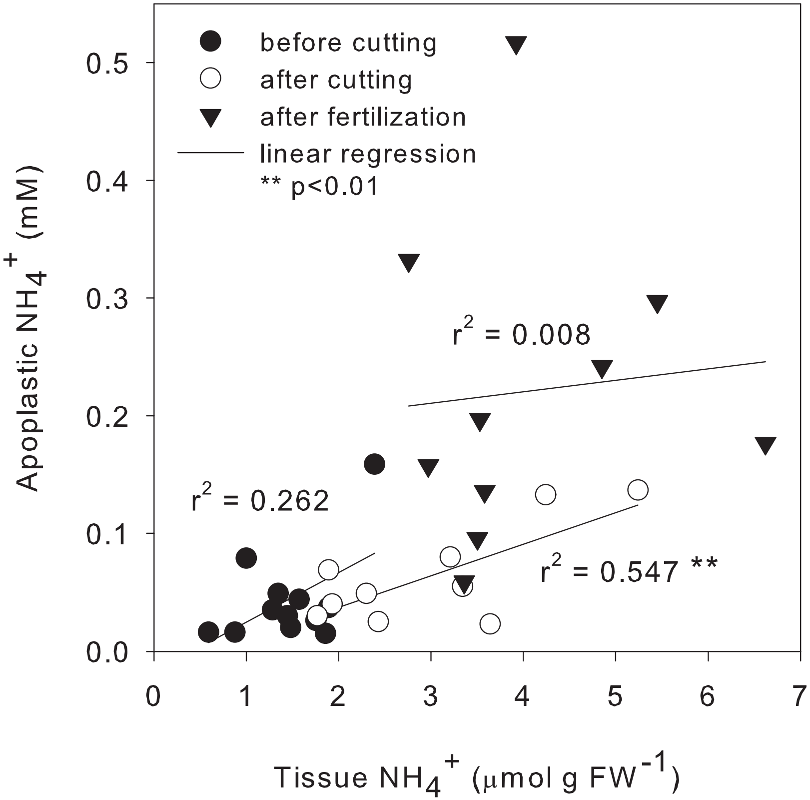

Fig. 6. Correlation between mean bulk leaf NH_4^+ and Γ (apoplastic NH_4^+/H^+) in leaves of a grass mixture during a diurnal course before and after cutting and after fertilization. **Significance at p<0.01.

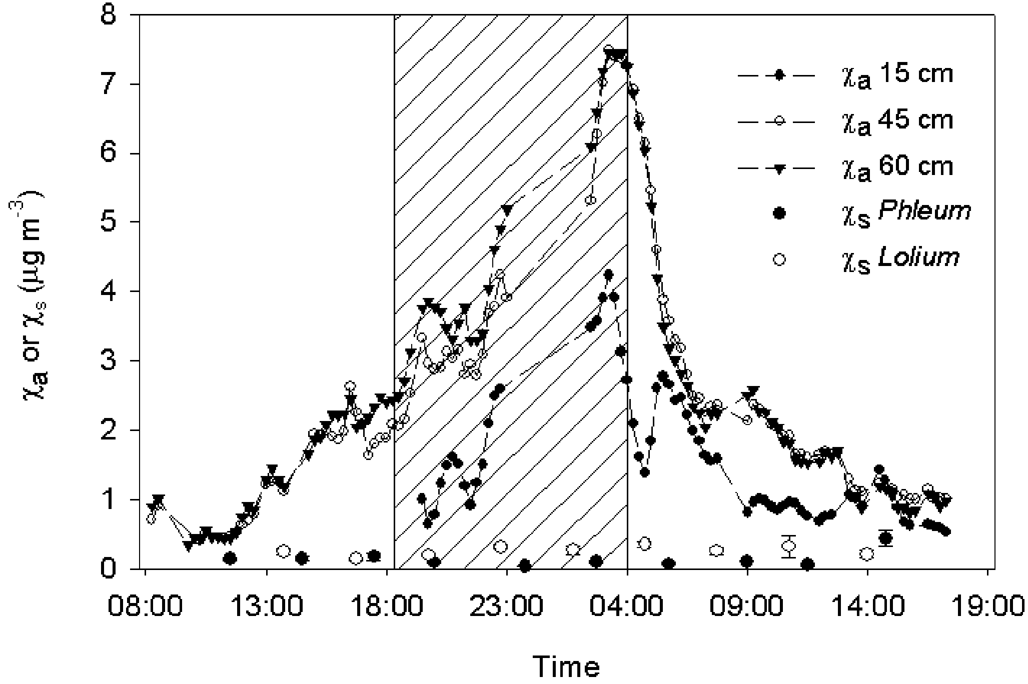

Fig. 7. Diurnal course of NH_3 flux above the plant canopy **(A)**, in-canopy NH_3 gradient and calculated χ_{NH_3} for the dominant grass species *Lolium perenne* and *Phleum pratense* **(B)** before cutting (26/27 May). The height of the canopy was 70 cm at this stage. χ_{NH_3} are means of 4 replicates±SE. The dark period is indicated by the shaded area.

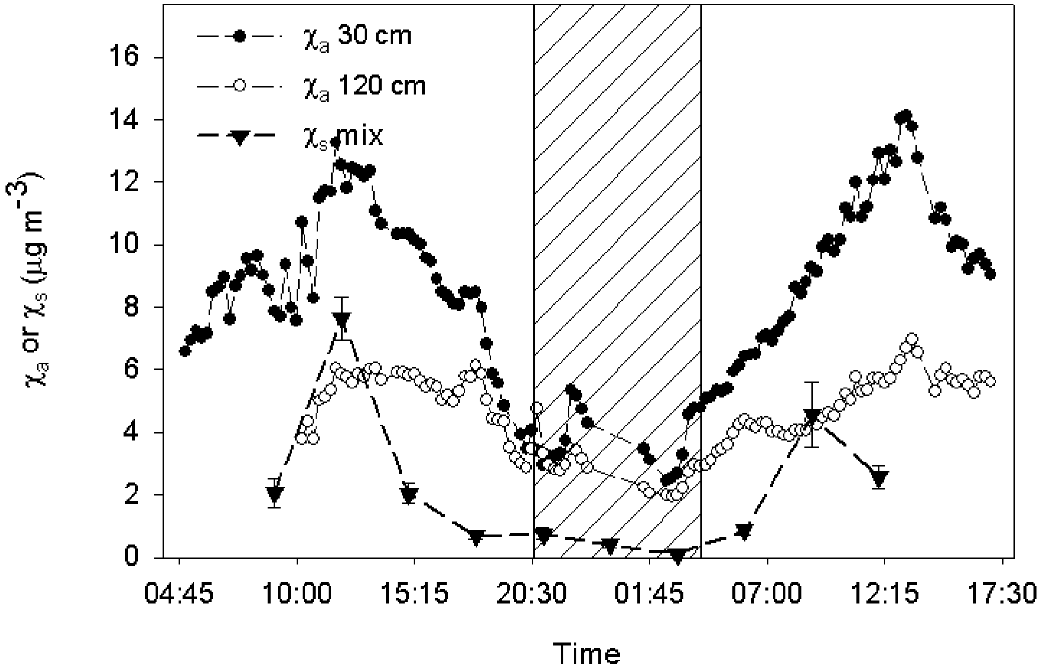

Fig. 8. Diurnal NH_3 gradient above the canopy and calculated χ_{NH_3} for grass stubbles after cutting and prior to fertilization (4/5 June). χ_{NH_3} are means of 4 replicates±SE. The dark period is indicated by the shaded area.

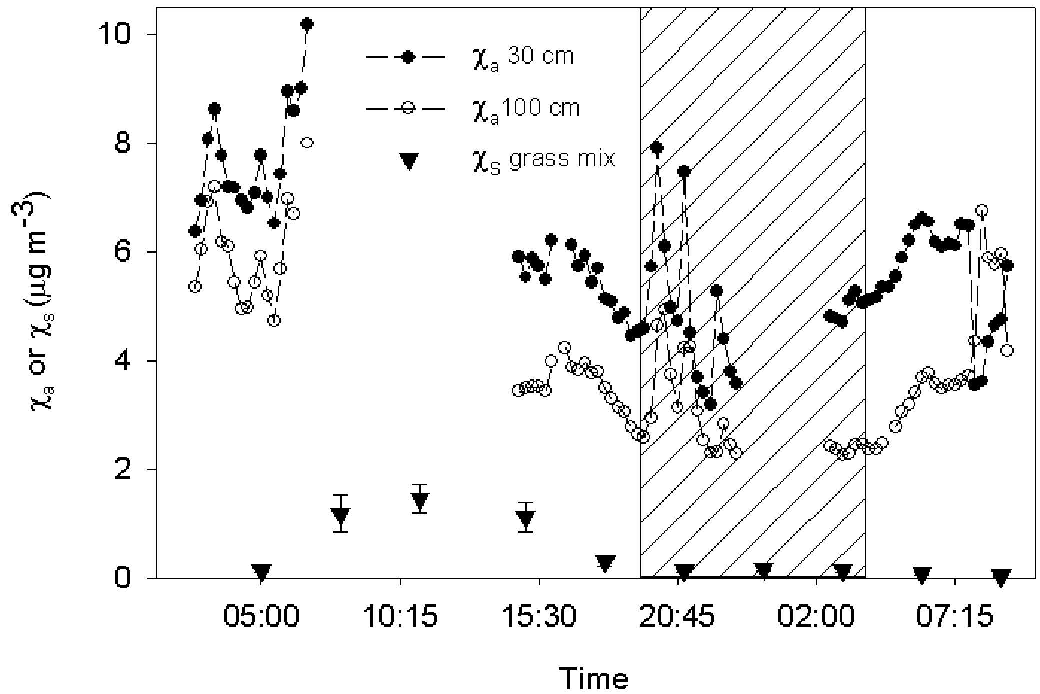

Fig. 9. Diurnal NH_3 gradient above the canopy and calculated χ_{NH_3} for grass stubbles 7 days after fertilization (12/13 June). χ_{NH_3} are means of 4 replicates±SE. The dark period is indicated by the shaded area.