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REGULAR ARTICLE

Effects of herbivory on N-cycling and distribution of added $^{15}NH_4^+$ in N-limited low-alpine grasslands

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Abstract Many rangelands around the world are degraded by severe overgrazing with resulting loss of nutrients and reduced productivity. However, grazing may also increase nutrient cycling and enhance ecosystem productivity. The aim of this study was to determine effects of grazing on availability of nitrogen (N), sources of N utilized by plants and cycling and distribution of N at a low-alpine site, Southern Norway. The study was part of a sheep grazing experiment with three density levels of sheep (no sheep, 25 km^{-2} and 80 km^{-2}) since 2001. The N-content of plants was determined in June 2008, August 2008 and August 2009. Indirect effects of herbivory on sources of N and N-cycling were assessed by $\delta^{15}N$ natural abundance and the system's distribution of added $^{15}NH_4$-N. We found little evidence for grazing induced effects on availability, sources or cycling of N based on N content of plants and $\delta^{15}N$ natural abundance. The organic soil horizon was the largest sink for the added $^{15}NH_4$-N. Proportional tracer recoveries and tracer enrichments indicate a somewhat greater N cycling at grazed than at non-grazed sites. We conclude that the experimental levels of grazing have limited impact on distribution and cycling of N and thus represent sustainable ecosystem management in terms of N dynamics in the long-term.

Keywords Alpine system · Nitrogen cycling · Nitrogen availability · Grazing · ^{15}N-tracer · $\delta^{15}N$ natural abundance

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Introduction

Managed grazing systems cover more than 25% of the global land surface (Asner et al. 2004). These systems occupy bioclimatically and edaphically marginal lands throughout much of the world (Asner et al. 2004). This is also the case in Norway, where there is a long history of using marginal lands for low intensity grazing (Austrheim et al. 2008a; Olsson et

al. 2000; Warren 1998). Although the number of sheep has remained relatively stable in Norway since 1950, the proportion of sheep grazing in mountain areas has increased (Austrheim et al. 2011). In 1999, 75% of all sheep in Norway grazed in the northern boreal and alpine region (Austrheim et al. 2008a). Nitrogen is the key limiting nutrient for plant growth in terrestrial ecosystems (Vitousek and Howarth 1991). Hence, changes in N availability caused by grazing may have severe implications for plant growth and species distribution. Despite the ecological and economic importance of these unfertilized, semi natural ecosystems, detailed investigations about effects of different grazing intensities on belowground nitrogen (N) dynamics are rare.

Grazing by large herbivores may have severe impacts on ecosystem structure (van der Wal et al. 2004; van der Wal and Brooker 2004) through direct and indirect impacts on ecosystem processes and properties important for N-cycling (Ewing et al. 2010; Frank 1998; Frank et al. 2000; Frank and Evans 1997; van der Wal and Brooker 2004). Direct impacts of herbivory include trampling, plant defoliation and nutrient return in form of urea and faeces (McNeill and Unkovich 2007; van der Wal et al. 2004). In turn, this may affect soil properties (e.g. temperature, moisture and bulk density), vegetation cover (e.g. species composition, biomass and nutritional status) as well as recycling and losses of N (De Deyn et al. 2009; Ewing et al. 2010; Martinsen et al. 2011b; McNeill and Unkovich 2007; van der Wal and Brooker 2004). According to Wardle et al. (2004) and Harrison and Bardgett (2008) the return of labile faecal material and input of litter with a high N-content in fertile systems may have positive effects on soil biota and thus nutrient cycling and N supply to plants. In turn, this will cause positive feedbacks favoring fast growing plants and hence retard succession.

Plant uptake and N concentrations of plant tissue reflect N concentrations in the soil solution, rates of N mineralization (Detling 1998; Hobbie and Gough 2002; McNeill and Unkovich 2007) and species specific adaptations to N uptake (Bradshaw et al. 1964; Gigon and Rorison 1972). However, the availability of soil N and plant tissue N concentrations may vary with season (Elberling et al. 2008; Morecroft et al. 1992a; Morecroft et al. 1992b).

The natural abundance of ^{15}N provides information about N-cycling (Högberg 1997; Robinson 2001; Xu et al. 2010), since several key processes in N-cycling involve isotope fractionation [e.g. NH_3 volatilization, nitrification and denitrification (Robinson 2001)]. Thus, $\delta^{15}N$ of soils and plants has been used as an indicator of the impact of various drivers on turnover and availability of N (Frank et al. 2000; Frank and Evans 1997; Garten et al. 2007; Högberg et al. 1996; Hyodo and Wardle 2009; Makarov et al. 2008). Isotopic enrichment (increased $\delta^{15}N$ of total soil-N) with soil depth is found in forests (Högberg et al. 1996) and in alpine/tundra zones of mountain areas (Makarov et al. 2008). Removal of above ground plant material depleted in ^{15}N as well as mixing of ^{15}N enriched deeper soil layers with surface horizons may increase $\delta^{15}N$ (Högberg 1997) of the latter. Hence, biomass removal and trampling associated with grazing may, in addition to grazing induced stimulation of N cycling, increase $\delta^{15}N$ of O-horizons and eventually of plants.

N-cycling may also be studied using ^{15}N-enriched tracers (Ewing et al. 2010; Näsholm et al. 1998; Providoli et al. 2005; Rütting et al. 2010). Ewing et al. (2010) clearly show the importance of detritus and soil moisture in controlling movement of N in Yellowstone grasslands. Despite a lack of grazing induced differences in plant ^{15}N, they found a greater retention of added ^{15}N in litter from historically ungrazed compared to grazed sites due to greater litter pools at ungrazed sites. The study clearly highlights the importance of litter being a key regulator of N flow. To our knowledge, little research has been done in low alpine, unfertilized systems with low N-deposition to determine density dependent effects of grazing on movement of inorganic N in different ecosystem components.

The main aim of this study was to determine long term (8 year) effects of grazing on (1) plant availability of N, (2) sources of N taken up by plants, (3) cycling of N and (4) distribution of N in low alpine grasslands using an experimental design with three density levels of domestic sheep (no sheep, low; 25 km^{-2} and high; 80 km^{-2}). We measured seasonal differences in total N-concentration of four plants (*Alchemilla alpina*, *Vaccinium myrtillus*, *Avenella flexuosa*, *Nardus stricta*) at each sheep density level as an indirect measure of effects of grazing on N availability in soils. Effects of grazing on N sources taken up by plants were determined using $\delta^{15}N$ natural abundance. Grazing induced effects on cycling

of N was determined indirectly using $\delta^{15}N$ natural abundance of O-horizons and recovery of added tracer N (applied as $^{15}NH_4Cl$). Effect of grazing on distribution of N was assessed using the system's distribution of added ^{15}N tracer in different soil and plant components.

We hypothesize total N-concentrations of plants to increase in the order no grazing < low grazing intensity < high grazing intensity (**H1a; testing main effect of grazing**) and to be greater early vs. late in the growing season (**H1b; testing main effect of season**). $\delta^{15}N$ natural abundance of all ecosystem components are expected to increase with increasing grazing intensity (**H2a; testing main effect of grazing**) due to ammonia volatilization of faeces, removal of depleted above-ground biomass and mixing of O-horizon and mineral soil at grazed sites. Belowground components are expected to have greater $\delta^{15}N$ natural abundances than above ground components (**H2b; testing main effect of component**). Assuming no grazing induced difference in species specific physiological N-uptake mechanisms we predict tracer recovery to increase in order high grazing intensity < low grazing intensity < no grazing (**H3a; testing main effect of grazing**) due to greater dilutions of added ^{15}N at grazed sites, which we hypothesize have a greater cycling of N. Differences between grazing levels in ^{15}N enrichment (expressed as ^{15}N atom% excess) are expected to decline with increased time since tracer application (**H3b; testing interaction between grazing and time since tracer application**).

Material and methods

Site description

The study was conducted in a low alpine region (1050–1320 m a.s.l.) in Hol municipality, Buskerud county, southern Norway (7°55′–8°00′ E, 60°40′–60° 45′ N) (Mysterud and Austrheim 2005). Mean annual temperature (MAT) is –1.5°C and mean annual precipitation (MAP) is about 1000 mm (Evju et al. 2009), approximately 75% of which falls as snow. The bedrock consists of meta-arkose and quaternary deposits of till and colluvium (Kristiansen and Sollid 1985; Sigmond 1998). Soils are freely drained with shallow and acidic organic horizons. Vegetation is dominated by dwarf shrub heaths with smaller patches of lichen heaths, snow beds and alpine meadow communities in lee-sides (Rekdal 2001). Further description of plant species composition is given by Austrheim *et al.* (2005).

In 2001 a grazing experiment (within a large enclosure; ~2.7 km^{-2}) with three treatments of domestic sheep (*Ovis aries*); no sheep (control), low grazing density (25 sheep km^{-2}) and high grazing density (80 sheep km^{-2}) was established (Mysterud et al. 2005; Mysterud and Austrheim 2005). The experiment is set up as a randomized block design with three blocks, each divided in three sub-enclosures (~0.3 km^{-2}) randomly assigned to each of the three grazing treatments (Mysterud et al. 2005; Mysterud and Austrheim 2005). Sheep grazing occurs from the end of June to the beginning of September each year (since 2002).

Previous findings documented a marked decline of plant N in the course of the growing season (Mysterud et al. 2011). Mysterud et al. (2011) also found the effect of growing season to interact with grazing (most strongly for *Avenella flexuosa*) with a smaller decline of N towards the end of the season at high sheep density as compared to controls without sheep. The impacts of grazing on N-content of the plants in late stages of the season was attributed to grazers keeping grasses in a young phenological stage (Mysterud et al. 2011). Furthermore, the system has a strong N limitation as observed by small concentrations of inorganic N and low potential mineralization rates in O-horizons (Martinsen et al. 2011a). However, potential mineralization rates were significantly greater in areas with high sheep density as compared to low sheep density and no sheep. Clear effects of grazing impact on bulk density (BD) and soil organic carbon (C) storage was reported by Martinsen et al. (2011b).

In June 2008, we established three adjacent 1 m × 1 m plots within each of the 9 sub-enclosures (i.e. three plots within each of the three sub-enclosures per block; a total of 3 × 3 × 3=27 plots). The plots were selected based on criteria of similar altitude (mean altitude 1168 m a.s.l) and plant community (Martinsen et al. 2011a). All plots were located in grassland habitats partly covered with willow-shrubs due to preferential grazing in these habitats (Mobæk et al. 2009). The plots were fenced-off during the summer 2008 to eliminate herbivore induced effects on phenology; cf.

Mysterud et al. (2011). The sampling of soil and vegetation was conducted in June and August 2008 and in August 2009.

^{15}N natural abundance: soil and vegetation sampling

Prior to tracer addition (26th of June to 2nd of July 2008), soil from the O-horizon and aboveground plant tissue (only fully developed individuals) of *A. flexuosa, N. stricta* and *A. alpina* were sampled adjacent to the 27 plots. Two O-horizon soil cores were sampled adjacent to the plots within each of the 9 sub-enclosures, using an auger (diameter 2.5 cm) to a maximum depth of 10 cm. The vegetation was cut at the soil surface and the litter (O_i) removed. Within each enclosure, both soil cores and vegetation samples collected adjacent to the plots were bulked prior to determination of ^{15}N natural abundance. Thus for each enclosure we have one value for natural abundance for the soil and each of the four plant species, so that the number of analyzed samples for the soil and each of the plant species was $n_{soil}=9$, $n_{A.\ flexuosa}=9$, $n_{N.\ stricta}=9$ and $n_{A.\ alpina}=8$ (absent in one enclosure). In addition, *Vaccinium myrtillus* and an integrated sample of cryptogams, roots and O_i (herafter called surface layer) was sampled outside each of the 27 plots September 10th and September 20–21 2009, respectively. *Vaccinium myrtillus* was sampled and bulked within each enclosure in the same way as the plants in 2008 ($n_{V.\ myrtillus}=8$, absent in one enclosure). The average volume of surface layer ($n_{surface\ layer}=27$) was 75.8 cm^3 (42.1 cm^2 × 1.8 cm).

Addition of ^{15}N tracer

The ^{15}N tracer was added to all 27 (1 m × 1 m) plots in July 2008 (9th–11th). Prior to the ^{15}N addition, the plots received 2 L of stream water (applied using a backpack sprinkler) to pre-wet the system. Next, 30.56 mg N m^{-2} (as ammonium chloride (NH_4Cl) 99.98 Atom % ^{15}N; IN 5037, Icon Isotopes, USA) in 5 L of stream water (6.11 mg N l^{-1}) were slowly added (to prevent surface runoff) using a 1 m long pvc tube (Φ 13 mm) with 10 small holes, each separated by 10 cm (a total of 100 addition points per m^{-2}). The pvc tube with the holes (connected to a backpack sprinkler) was inserted below the vegetation, on top of the litter layer to prevent contamination of the vegetation. The N concentration of the stream water was low (0.08 mg N l^{-1}, (Martinsen et al. 2011a), and no corrections for the ^{15}N from the stream water were made. After tracer addition, the plots received additional 3 L of stream water from a backpack sprayer to wash off ^{15}N from the leaves in case of contamination. The added N represents about 7.3% of the annual N deposition (Aas et al. 2008) and was assumed not to affect the N cycling in the system. At some plots, minor surface runoff was generated during application, which will have resulted in tracer loss. Thus, the percentage tracer recovery may in some cases be underestimated.

Tracer addition experiment: soil and vegetation sampling

Chemical and physical attributes in addition to number of samples from the tracer addition experiment are presented in Online Resources 1, 2 and 3. After tracer addition, the O-horizon and aboveground plant tissue of *A. flexuosa, N. stricta and A. alpina* were sampled at two occations (12th–13th of August 2008 and 2nd–4th of August 2009) inside the 27 plots. In 2008, two randomly located O-horizon soil cores (vegetation and O_i removed) were sampled at each plot using an auger (diameter 5.2 cm) to a maximum depth of 10 cm (and bulked per plot prior to analysis; $n_{soil}=27$). The number of samples of each of the plant species used for analysis was $n_{A.\ flexuosa}=27$, $n_{N.\ stricta}=21$ and $n_{A.\ alpina}=19$ (Online Resource 2 and 3).

In 2009, all aboveground vegetation within a sub-area (0.5 m × 0.5 m) in the middle of each of the 27 plots was harvested quantitatively. The harvested plant shoots were sorted at the species level; *A. flexuosa* ($n=27$), *N. stricta* ($n=18$, only 17 were used for chemical analysis), *A. alpina* ($n=14$) and *V. myrtillus* ($n=21$) (Online Resource 2 and 3). These species were selected due to their most frequent presence at the plots. The rest of the vegetation was divided into six compartments, including ferns, rest of herbs, rest of woody species, rest of cryptogams, rest of graminoids and non-recognizable or withered plant material ("litter mix") to get an esimate of the total aboveground biomass (Online Resource 1). Three O-horizon soil cores (vegetation and O_i removed) randomly located within each sub-area of the 27 plots were sampled using an auger (diameter 2.5 cm; bulked per plot prior to analysis; $n_{soil}=27$). Finally, three surface layer samples (randomly

located within each sub-area) were sampled (the same procedure as before tracer addition) and bulked prior to analysis ($n_{surface\ layer}$=27). The average volume of surface layer sampled was 162.5 cm^3 (108.3 cm^2 × 1.5 cm).

Analysis and laboratory processing

All soil and surface layer samples were stored dark and cold (<4°C) prior to analysis. Soils were air-dried (40°C for 4–5 days) and sieved at 2 mm to separate the dry roots and gravels from the soil. The weight of dry roots and gravels (>2 mm) was determined and the roots included for chemical analysis (representing bulked roots of different plant species). Sub samples of the air-dried fine earth fraction were dried at 105°C to determine dry matter content (DM), while soil samples from August 2008 were dried at 60°C. Vegetation and surface layer samples were air-dried (40°C for 4–5 days) and homogenized. The above-ground biomass (g) was calculated as the air dry weight of each aboveground vegetation compartment (see above) divided by the area sampled (0.25 m^2). Bulk density (g cm^{-3}) was based on the fine earth (DM corrected) fraction of the soil. The mass of the roots (>2 mm) per unit of soil volume (g cm^{-3}) was calculated as the air dried weight of roots divided by the volume of soil sampled. Furthermore, density (g cm^{-3}) of the surface layers was calculated as the air dried weight of the surface layers divided by the volume sampled. pH of the soil (~ 4.4, Online Resource 3) was determined electrometrically (Orion, model 720) in a soil solution with distilled water (volume soil : volume water ratio 1:2.5) (Krogstad 1992).

Air dried subsamples of the O-horizon (O_e+O_a), soil roots from O_e and O_a, surface layers and plant tissue (four species) were ground in a ball mill (Fritsch, pulverisette, type 05002) prior to analysis of total C and N content and ^{15}N isotopic composition both for the natural abundance and the ^{15}N labelling experiment. The analyses were conducted at The Macaulay Land Use Research Institute, Aberdeen, UK. The total N contents and the ^{15}N:^{14}N isotope ratios of the milled dried material were determined using a Flash EA 1112 Series Elemental Analyser connected via a Conflo III to a Delta^{Plus} XP isotope ratio mass spectrometer (all Thermo Finnigan, Bremen, Germany). The isotope ratios were calculated with respect to N_2 reference gasses injected with every sample and traceable to IAEA reference materials USGS40 and USGS41 (both L-glutamic acid). The N contents of the samples were calculated from the area output of the mass spectrometer calibrated against National Institute of Standards and Technology (NIST) standard reference material 1547 peach leaves which was analysed with every batch of ten samples. Long term precision for quality control standards (milled flour) were: total N=1.7±0.04% and ^{15}N:^{14}N=0.367± 0.0002 atom % (mean±sd, n=200).

Calculations

The isotopic values of ^{15}N natural abundance are reported as

$$\delta^{15}N = 1000*\left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \quad (1)$$

where $\delta^{15}N$ is expressed in ‰, R is the ^{15}N:^{14}N isotope ratio and $R_{standard}$=0.0036765 (atmospheric N_2; $\delta^{15}N$=0‰) (Robinson 2001). ^{15}N atom% excess was calculated as

$$^{15}N_{atom\%excess} = \left(\frac{Atom\%_{enriched} - Atom\%_{background}}{Atom\%_{background}}\right)*100 \quad (2)$$

We assumed negligible N isotope fractionations during tracer movement in the system (Robinson 2001) and no difference in ^{15}N natural abundance within the pools from 2008 to 2009. The mass of recovered ^{15}N in each pool (mg m^{-2}) was calculated as

$$Mass^{15}N = \left(X_{sample}*N_{content}*Mass\right)*10^3 \quad (3)$$

where X_{sample} is the tracer fraction in the samples, calculated according to (Providoli et al. 2005):

$$X_{sample} = \frac{\left(F_{sample} - F_{reference}\right)}{\left(F_{tracer} - F_{reference}\right)} \quad (4)$$

$N_{content}$ is the concentration of total N per unit biomass, surface layer or soil (g g^{-1}), mass (g m^{-2}) is the total mass of each pool (biomass=total dry weight (g) per m^{-2}; surface layer, soil and roots=density (g cm^{-3}) * depth (cm) * 10^4) and 10^3 is a conversion factor from g to mg. F_{sample} is the fractional abundance of ^{15}N in the sample (i.e. [^{15}N/(^{15}N +^{14}N)]*100= atom% ^{15}N) and F_{tracer} and $F_{reference}$ is the fractional

abundance of ^{15}N in the applied tracer (99.98 atom % ^{15}N) and in the non-labelled samples (i.e. natural abundance), respectively. The percentage tracer recovered ^{15}N in each pool was calculated as the mass of recovered ^{15}N in each pool divided by the amount of added tracer (30.56 mg Nm^{-2}).

To account for differences in tracer recovery between plots (reported as proportional recovery), the total recovery at each plot (n=27) was rescaled to 100% (Finzi and Berthrong 2005), which gives the proportional partitioning of added ^{15}N in the different ecosystem pools (i.e. vegetation, surface layer, O-horizon and roots) (Clemmensen et al. 2008).

Statistics

Statistical analyses were conducted using the libraries lme4 and multcomp in the statistical package R (version 2.10.1) (R Development Core Team 2009). We used linear mixed effects models with random effects reflecting the block-wise randomization design. The random effects always included blocks (3 levels) and three sub-enclosures nested within each block (n=9). In some cases, not all sub-enclosures were included due to missing data (Online Resource 4). According to H1a and H1b the N-content (Fig. 1) of each plant species was tested focusing on the main effect of grazing treatment and sampling month, respectively. The $\delta^{15}N$ natural abundance (Fig. 2) was tested focusing on the main effect of component (7 levels; *A. alpina*, *V. myrtillus*, *A. flexuosa*, *N. stricta*, surface layer, O-horizon and roots) and grazing treatment (H2a and H2b). In addition, species specific responses of grazing on sources of N taken up by the plants was tested by a treatment*component interaction (Online Resource 4). Proportional tracer recovery (Fig. 3) was tested for the effect of grazing treatment (H3a) per gram of plant N m^{-2}. We assumed no grazing induced difference in species specific physiological N-uptake mechanisms. The analysis were conducted on separate species due to potential differences in rooting depth and source of N taken up by the plants, both which may affect the degree of dilution of the added tracer. Furthermore, the calculated ^{15}N

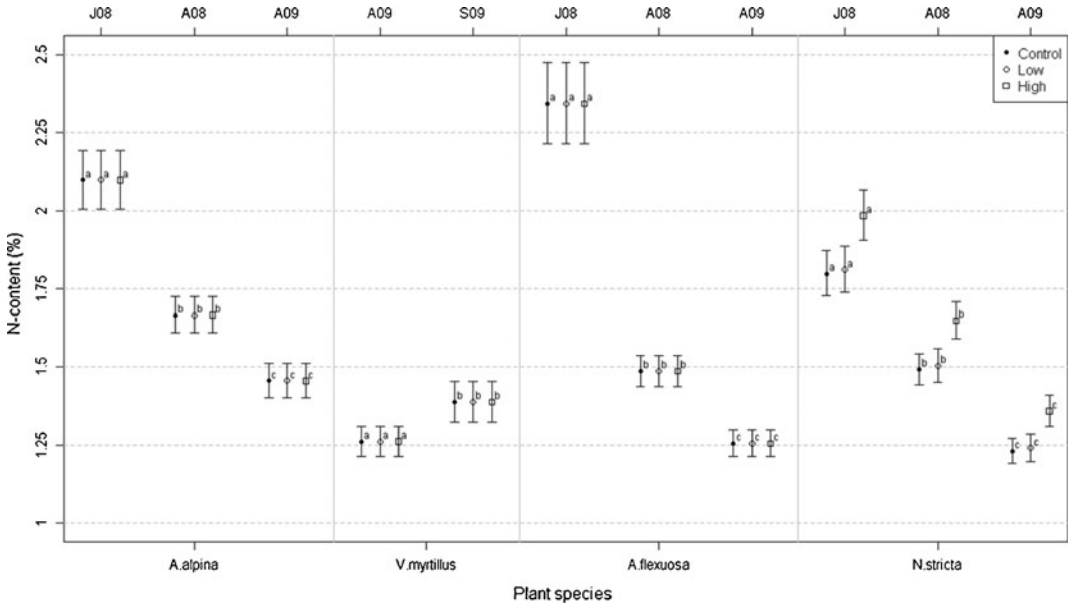

Fig. 1 Estimated mean (± se) N-content (% total-N) of *A. alpine* (n=41), *V. myrtillus* (n=29), *A. flexuosa* (n=63) and *N. stricta* (n=44) from 27 grassland habitats subjected to different grazing densities of sheep (control=no sheep, low=25 sheep km^{-2} and high=80 sheep km^{-2}) at Hol, Norway. The figure shows estimated N-contents of plots with no ^{15}N addition (June 2008; J08 and September 2009; S09), 1 month (i.e. August 2008; A08) and 13 months (i.e. August 2009; A09) after ^{15}N addition (July 2008). Estimates are based on linear mixed effect models presented in Online resource 4. Different letters indicate difference at the level of significance <0.05. **Note:** *V.myrtillus* and surface layer were sampled in 2009 for determination of ^{15}N natural abundance (see : "Material and methods")

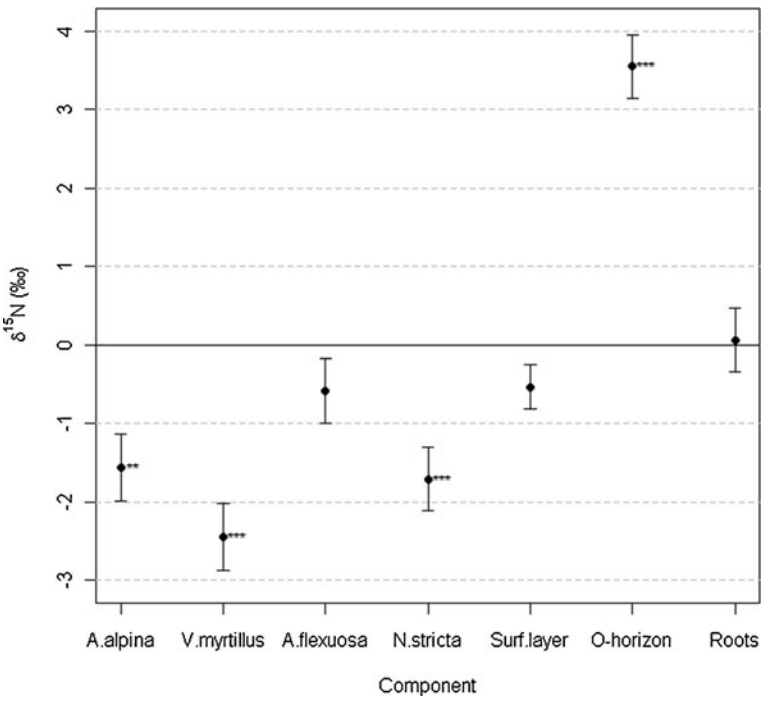

Fig. 2 Estimated mean δ^{15}N (‰) natural abundance (± se) of above- and belowground components (sampled prior to tracer addition) from 27 grassland habitats, Hol, Norway. Values derive from a linear mixed effect model with component (7 levels; *A. alpina*, *V. myrtillus*, *A. flexuosa*, *N. stricta*, surface layer, O-horizon and bulked plant roots >2 mm) as the only fixed effect factor (Online resource 4). ** and *** indicate significant difference from $\delta^{15}N=0$ at the level of significance 0.01 and 0.001, respectively. Positive δ^{15}N values indicate enrichment and negative δ^{15}N values indicate depletion of ^{15}N relative to atmospheric N_2 ($\delta^{15}N=0$). $n=79$

atom% excess and proportional recoveries were correlated (results not shown). Due to lack of biomass data for 2008, we used ^{15}N atom% excess (Fig. 4) to assess differences in ^{15}N enrichment between the grazing treatments short term (1 month) and long term (13 months) after tracer addition (i.e. focusing on the interaction between month and treatment; H3b).

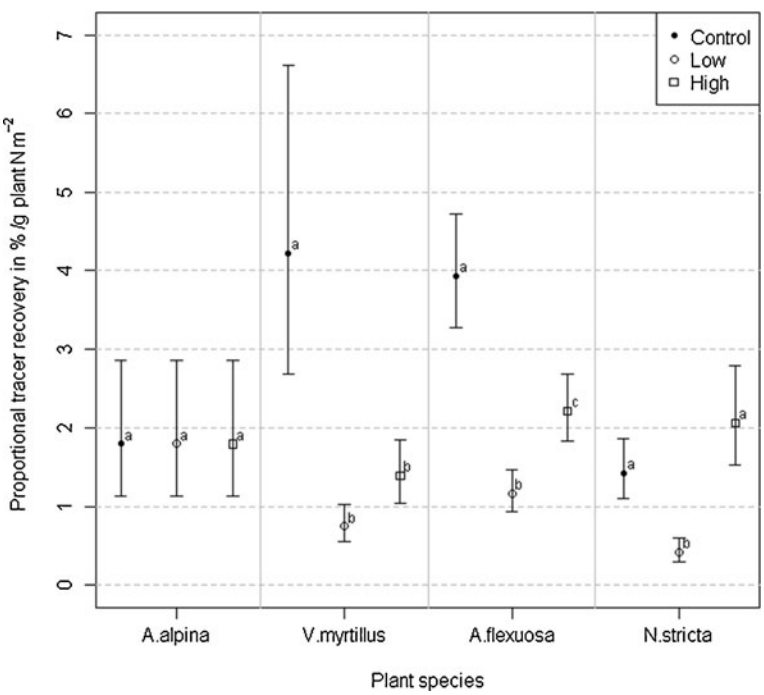

Fig. 3 Estimated mean (± se) proportional tracer recovery as % of recovered tracer per g plant N-pool and m^2, 13 months after ^{15}N addition (August 2009) for *A. alpina* ($n=14$), *V. myrtillus* ($n=20$), *A. flexuosa* ($n=26$) and *N. stricta* ($n=17$) from 27 grassland habitats at different grazing densities of sheep (control= no sheep, low=25 sheep km^{-2} and high=80 sheep km^{-2}), Hol, Norway. Values derive from linear mixed effect models (Online resource 4). Different letters indicate difference at the level of significance <0.05

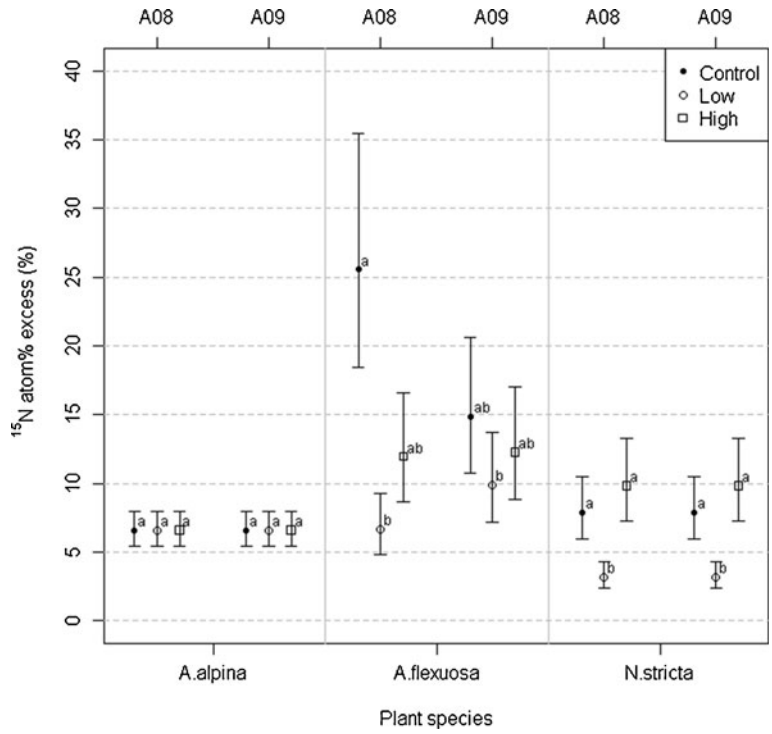

Fig. 4 Estimated mean (± se) ^{15}N atom% excess (percentage increase in atom% ^{15}N of enriched samples relative to the background atom% ^{15}N) of *A. alpina* (n=33), *A. flexuosa* (n=54) and *N. stricta* (n=38) 1 month (i.e. August 2008; A08) and 13 months (i.e. August 2009; A09) after ^{15}N addition to 27 grassland habitats at different grazing densities of sheep (control=no sheep, low=25 sheep km^{-2} and high=80 sheep km^{-2}), Hol, Norway. Values derive from linear mixed effect models for each plant species (Online resource 4). Different letters indicate difference at the level of significance <0.05. **Note**: *V. myrtillus* is not included because not sampled in August 2008. ^{15}N atom% excess was used instead of proportional tracer recovery for the comparisons between 2008 and 2009 due to lack of data on plant biomass in 2008

Backward selection was used [models fitted by maximum likelihood (ML)] and models compared based on AIC ("smaller is better") and likelihood ratio tests (Chi squared) to obtain the minimum adequate model. The best model was re-fitted based on restricted maximum likelihood (REML) and the estimated effects (including se; Online Resource 4) were calculated using general linear hypothesis testing. Only adjusted p-values [single-step method (Hothorn et al. 2008)] are reported. All variables except δ^{15}N were ln transformed to avoid violations of the model assumptions (back-transformed before plotting). Residuals and predicted random effects were plotted (histograms and QQ normal plots) to assess normality and potential outliers. Any missing or omitted values are reported in Online resource 4.

Results

Nitrogen concentration

The total N concentrations (% N) of *A. alpina, A. flexuosa* and *N. stricta* were significantly smaller in August 2008 (i.e. 1 month after tracer addition; 1.5–1.7% N) than in June 2008 (prior tracer addition; 1.8–2.3% N), illustrating a seasonal decline in %N and the low impact of the added tracer N (30.56 mg N m^{-2}) (Fig. 1). A further decline was observed 13 months after tracer addition (i.e. August 2009). There was no effect of grazing except for a tendency of greater N contents for *N. stricta* at high density of sheep as compared to low density and control treatments (Fig. 1). By contrast, the N-concentration of the surface layer was significantly

greater at sites with high sheep density as compared to low sheep density and non-grazed sites. N-concentrations did not differ between grazing treatments for the O-horizon or the roots (Online Resource 4).

^{15}N natural abundance determined prior to tracer addition (June 08)

The surface layer (a mixture of cryptogams, roots and O_i), *A. flexuosa*, *A. alpina*, *V. myrtillus* and *N. stricta* were depleted (the 3 latter significantly depleted), and the O-horizon soil significantly enriched in ^{15}N relative to the atmospheric N_2 (Fig. 2, Table 1). The bulked plant roots within O-horizons had $\delta^{15}N$ values close to 0, i.e. somewhat between the O horizon and the plants (Fig. 2). Despite tendencies of greater $\delta^{15}N$ levels in plants, surface layer and O-horizon at the high grazing density (Table 1), the differences were not significant.

Tracer addition experiment: ^{15}N recovery 13 months after tracer addition

The total recovery of added tracer-N 13 months after tracer addition (August 2009) differed greatly between the ecosystem components and the grazing treatments (Table 2). The surface layer and the O-horizon were the largest sinks per unit surface area (>90% of the recovered tracer), illustrating the importance of these components for initial N-retention. Also, the proportional tracer recovery (i.e. percent recovery of each individual component normalized to the total amount of recovered tracer at each plot)

Table 1 Mean total carbon and nitrogen concentration (%), $\delta^{15}N$ natural abundance (‰) and sampling depth (cm) for different plant and soil components (sampled prior to tracer addition, i.e. June–July 2008) from 27 grassland habitats at different grazing densities of sheep (control=no sheep, low=25 sheep km^{-2} and high=80 sheep km^{-2}), Hol, Norway. Standard error (se) and number of replicates (n) is shown. "-" indicates no value

Treatment	Component	C conc.			N conc.			$\delta^{15}N$			Depth		
		(%)	se	n	(%)	se	n	(‰)	se	n	(cm)	se	n
Control	*Alchemilla alpina*	43.0	0.2	2	2.2	0.0	2	−1.72	0.36	2	–	–	–
	Vaccinium myrtillus[a]	47.4	0.2	3	1.4	0.0	3	−3.02	1.17	3	–	–	–
	Avenella flexuosa	42.4	0.2	3	2.5	0.1	3	−1.97	0.39	3	–	–	–
	Nardus stricta	42.0	0.5	3	1.9	0.1	3	−1.50	0.60	3	–	–	–
	Surface layer[a]	36.6	2.0	9	1.5	0.1	9	−0.77	0.30	9	1.8	0.2	9
	O-horizon	23.3	6.7	3	1.1	0.2	3	2.91	1.73	3	2.5	0.2	3
	Roots	42.7	2.4	3	1.0	0.2	3	−0.48	0.33	3	2.5	0.2	3
Low	*Alchemilla alpina*	42.8	0.2	3	2.1	0.2	3	−2.20	0.65	3	–	–	–
	Vaccinium myrtillus[a]	47.7	0.6	2	1.3	0.0	2	−2.82	0.53	2	–	–	–
	Avenella flexuosa	42.1	0.1	3	2.4	0.3	3	0.23	0.71	3	–	–	–
	Nardus stricta	41.7	0.2	3	1.7	0.1	3	−2.29	0.15	3	–	–	–
	Surface layer[a]	39.4	1.8	9	1.6	0.1	9	−0.51	0.29	9	2.2	0.6	9
	O-horizon	22.8	2.2	3	1.4	0.0	3	3.80	0.81	3	4.9	2.3	3
	Roots	40.9	1.7	3	0.9	0.1	3	0.49	0.64	3	4.9	2.3	3
High	*Alchemilla alpina*	42.9	0.1	3	2.0	0.0	3	−0.77	0.12	3	–	–	–
	Vaccinium myrtillus[a]	47.4	0.1	3	1.4	0.0	3	−1.69	0.35	3	–	–	–
	Avenella flexuosa	42.4	0.3	3	2.2	0.1	3	−0.01	0.54	3	–	–	–
	Nardus stricta	42.3	0.2	3	2.0	0.1	3	−1.34	0.58	3	–	–	–
	Surface layer[a]	38.0	2.2	9	1.7	0.0	9	−0.33	0.41	9	1.5	0.1	9
	O-horizon	19.9	8.2	3	1.1	0.3	3	3.93	1.07	3	3.2	0.8	3
	Roots	42.7	1.0	3	1.0	0.1	3	0.17	0.22	3	3.2	0.8	3

[a] Sampled 2009 (see "Material and methods")

Table 2 Mean pool of total nitrogen (N pool), percentage fractional abundance of added ^{15}N tracer (X sample), recovered mass of added ^{15}N tracer (mass ^{15}N), mean percentage recovery of total added ^{15}N tracer within each treatment and component combination (recovery) and proportional recovery (i.e. percent recovery of each component relative to the total recovery at each plot) of 7 plant and soil components from 27 grassland habitats at different grazing densities of sheep (control=no sheep, low=25 sheep km^{-2} and high=80 sheep km^{-2}), Hol, Norway. Samples are collected 13 months after ^{15}N tracer addition (August 2009). Standard error (se) and number of replicates (n) is shown. "-"indicates no value

Treatment	Component	N pool			X sample			Mass ^{15}N			Recovery			Proportional recovery		
		(g m^{-2})	se	n	(%)	se	n	(mg m^{-2})	se	n	(%)	se	n	(%)	se	n
Control	*Alchemilla alpina*	0.16	–	1	0.068	–	1	0.111	–	1	0.36	–	1	0.61	–	1
	Vaccinium myrtillus	0.20	0.05	6	0.063	0.201	6	0.090	0.015	6	0.30	0.05	6	0.74	0.19	6
	Avenella flexuosa	1.23	0.29	9	0.061	0.096	9	0.619	0.156	9	2.03	0.51	9	4.57	0.97	9
	Nardus stricta	0.31	0.12	7	0.033	0.063	7	0.091	0.042	7	0.30	0.14	7	0.55	0.19	7
	Surface layer	57.73	8.08	9	0.019	0.028	9	10.393	1.972	9	34.00	6.43	9	68.49	6.38	9
	O-horizon	50.91	7.81	8	0.009	0.025	9	3.659	0.843	8	11.98	2.75	8	26.17	4.59	8
	Soil roots	2.43	0.87	9	0.018	0.036	9	0.361	0.112	9	1.18	0.37	9	2.69	0.94	9
Low	*Alchemilla alpina*	0.09	0.08	5	0.025	0.071	5	0.035	0.033	5	0.12	0.11	5	0.13	0.12	5
	Vaccinium myrtillus	0.77	0.20	6	0.023	0.040	6	0.191	0.074	6	0.62	0.24	6	0.67	0.26	6
	Avenella flexuosa	0.24	0.08	9	0.039	0.046	9	0.110	0.045	9	0.36	0.15	9	0.39	0.14	9
	Nardus stricta	0.11	0.05	5	0.019	0.041	5	0.017	0.007	5	0.06	0.02	5	0.07	0.03	5
	Surface layer	66.44	3.79	9	0.031	0.054	9	19.835	3.205	9	64.92	10.49	9	67.65	7.40	9
	O-horizon	195.02	47.15	9	0.004	0.010	9	7.007	1.453	9	22.96	4.73	9	30.04	7.59	9
	Soil roots	2.02	0.31	9	0.017	0.026	9	0.353	0.077	9	1.15	0.25	9	1.37	0.35	9
High	*Alchemilla alpina*	0.62	0.18	8	0.034	0.091	8	0.148	0.053	8	0.48	0.17	8	0.85	0.36	8
	Vaccinium myrtillus	0.61	0.13	9	0.027	0.053	9	0.216	0.074	9	0.71	0.24	9	1.16	0.42	9
	Avenella flexuosa	0.57	0.10	9	0.048	0.065	9	0.265	0.062	9	0.87	0.20	9	1.33	0.32	9
	Nardus stricta	0.58	0.24	5	0.061	0.174	5	0.285	0.191	5	0.93	0.62	5	1.30	0.71	5
	Surface layer	65.77	5.51	9	0.024	0.046	9	15.173	3.306	9	49.66	10.81	9	65.19	6.78	9
	O-horizon	115.97	35.43	9	0.006	0.011	9	5.871	1.598	9	19.18	5.19	9	28.17	6.37	9
	Soil roots	3.18	0.67	9	0.015	0.022	9	0.472	0.126	9	1.54	0.41	9	2.67	0.88	9

revealed greatest recovery in these two components, but no significant effect of grazing (Table 2 and Online Resource 4).

There was a large variation (0.07–4.6%) in the proportional tracer recovery for *A. alpina*, *V. myrtillus*, *A. flexuosa* and *N. stricta* 13 months after tracer addition (Table 2). The proportional tracer recovery was positively related to the plant N-pool per unit of surface area and (expressed per gram of plant N m^{-2}) revealed significant effects of grazing for *V. myrtillus*, *A. flexuosa* and *N. stricta* (Fig. 3). Proportional tracer recoveries were significantly greater at the sites with no sheep grazing (4.22 and 3.93%) compared the high (1.39 and 2.22%) and low (0.75 and 1.17%) grazing treatments for *V. myrtillus* and *A. flexuosa,* respectively. For *A. flexuosa* and *N. stricta*, proportional recoveries were significantly smaller at the low as compared to the high grazing treatment and the controls with no sheep (Fig. 3).

Tracer addition experiment: temporal change in distribution of added tracer (1 month vs. 13 months after addition)

The tracer enrichment of *A. alpina* (expressed as ^{15}N atom% excess) did not differ significantly between 1 and 13 months after tracer addition and was not affected by grazing (Fig. 4). By contrast, there was a significant interaction between time after tracer application and grazing treatment for ^{15}N atom% excess in *A. flexuosa*, with greater differences between the control as compared to the high and the low grazing treatments in 2008 than 2009 (Fig. 4). ^{15}N atom% excess in *N. stricta* was

significantly affected by grazing, but did not differ between the years (Fig. 4).

Discussion

We found no evidence of grazing induced differences in N-content (and CN ratio; not shown) of the plant species *A. alpina*, *V. myrtillus* and *A. flexuosa* assessed in this study (H1a), if effects of delayed phenology at grazed sites were prevented. However, we found evidence for somewhat greater N contents in *N. stricta* at high sheep density (Fig. 1). If grazing induced delayed phenology was allowed to occur (i.e. grazers keeping grasses in a young phenological stage), Mysterud et al. (2011) showed at the same experimental site that grazing had a significant effect on N-content of the grasses *A. flexuosa* and *A. odoratum*. As has been reported by Morecroft et al. (1992b) and Mysterud et al. (2011), N-content of all species (except *V. myrtillus*) was significantly smaller at late stages of the growing season (H1b). Smaller nutrient concentrations in aboveground plant tissue at the end of the growing season are most likely explained by continued biomass production in the course of the growing season which dilutes plant N (Körner 2003; Morecroft et al. 1992b) and resorption of N from senescing tissue (Aerts 1996; Körner 2003; Morecroft et al. 1992b). Since all plants investigated in our study were protected from grazing, so the phenological stage between treatments was similar, the treatment-related differences in N-content observed by Mysterud et al.(2011) cannot be explained by N availability and must have been related to phenology.

According to Nagy and Grabherr (2009) plants exposed to increased availability of N not necessarily show increased N concentration but alternatively may respond with increased plant tissue production (i.e. no increase in tissue N-content). A coarse estimate of the N pool of the total standing biomass (Online Resource 1) revealed, however, no significant difference between the grazing treatments (results not shown). Possibly, grazing affected belowground N-pools which may be significantly greater than aboveground pools (Fisk et al. 1998). However, this was not confirmed by N-pools of roots within the O-horizons, which differed only slightly between the grazing treatments (Table 2).

There was a significantly greater N-concentration in the surface layer at high sheep density as compared to the sites with low sheep density and no sheep (Online Resource 4), which we assume is caused by contamination of urea and faeces. By contrast, there were no effects of grazing on N-concentrations in the O-horizon or in the roots.

The natural abundance of ^{15}N differed significantly between the ecosystem components with O-horizons being significantly enriched ($\delta^{15}N$ ~3.5‰) and plants significantly depleted ($\delta^{15}N$~−1.6‰) as compared to $\delta^{15}N$ of the atmospheric standard (H2b). Levels of $\delta^{15}N$ natural abundance in the plants varied from −2.5‰ (*V. myrtillus*) to −0.6‰ (*A. flexuosa*). Probably, the difference in $\delta^{15}N$ natural abundance between the plants is caused by species specific traits, as $\delta^{15}N$ abundance in plants depends on the source of plant N and the form of N used, both of which are related to mycorrhizal status and rooting depth (Högberg 1997; Michelsen et al. 1996; Nadelhoffer et al. 1996; Weigelt et al. 2005). Furthermore, as suggested by Högberg (1997), under N-limited conditions, characterized by low rates of nitrification (and thus little N fractionation), uptake of NH_4-N in plants would result in depleted N at the soil surface due to accumulation of ^{15}N depleted litter. Earlier, low nitrification potentials at Hol were reported by Martinsen et al. (2011a). They found small concentrations of NO_3-N in soil water (sampled *in situ* using macrorhizons), little available NO_3-N (assessed by PRS^{TM}-adsorption) and low potential nitrification rates in O-horizons. This further supports our earlier findings that loss of inorganic N is small at Hol. Minor amounts of N are leached from the system as dissolved organic N (Martinsen et al. 2011a).

Despite a tendency for greater $\delta^{15}N$ in the soil and plant components at the high grazing treatment (Table 1), effects were not significant, neither in the aboveground nor in the belowground components (H2a). In Yellowstone National Park, Frank and Evans (1997) found an increased soil $\delta^{15}N$ in ungulate urine and dung patches, which was related to enhanced N-loss via leaching, ammonia volatilization and/or denitrification. By contrast, ^{15}N in plants was reduced due to uptake of depleted NO_3^- compared to soil NH_4^+. A decrease in $\delta^{15}N$ in surface soils (0–2 cm) caused by grazing is reported by Xu et al. (2010) in a rangeland in Inner Mongolia. However, they found no significant effects of grazing

(five grazing intensities) on $\delta^{15}N$ in topsoils (0–10 cm) or in plants. Based on previous studies at Hol, where we found mixing of O-horizon and mineral soil due to grazing (Martinsen et al. 2011b), a greater $\delta^{15}N$ would have been expected at grazed sites due to trampling. In addition, there is evidence for a greater biomass removal at high sheep density, as lamb weights are reduced at high densities (Mysterud, unpublished material). Also, removal of depleted plant biomass by herbivores was expected to increase $\delta^{15}N$ of O-horizons and eventually of plants. As discussed by Evju *et al.* (2006), the high sheep density used in this experiment may correspond to a moderate grazing pressure. As we found only weak evidence for increased $\delta^{15}N$ natural abundance at the intensively grazed site, we suggest that the effects of grazing at the levels provided at Hol on N cycling and source of N utilized by plants are small.

Recovery of added tracer ^{15}N was greatest in the surface layer and O-horizon (Table 2). This is in accordance with other labeling experiments, indicating strong retention of N in these layers (Ewing et al. 2010; Gerzabek et al. 2004; Näsholm et al. 1998; Rütting et al. 2010). Furthermore, we found evidence for greater proportional recoveries per gram plant N (*V. myrtillus* and *A. flexuosa*) at non-grazed as compared to grazed sites (H3a). The proportional recovery per gram plant N was smallest at the low grazing treatment for *V. myrtillus*, *A. flexuosa* and *N. stricta* (Fig. 3, Online Resource 4), indicating a greater N-cycling (high immobilization-mineralization turnover) and thus ^{15}N dilution at low sheep density as compared to high sheep density and control. The somewhat greater N-cycling at intermediate sheep density may enhance the competitive ability of plants with rapid growth that are tolerant to grazing [e.g. graminoids (Coughenour 1985)] in the long term. Sheep at low density may thus indirectly facilitate their own food supply (Augustine and McNaughton 1998).

The difference in ^{15}N atom% excess between the grazing treatments decreased with time (i.e. 1 month vs. 13 months) after tracer addition, but for *A. flexuosa* only (Fig. 4). This suggests reduced effects of grazing on ^{15}N dilution with increased time since tracer application (H3b) probably due to stabilization of ^{15}N in the soil organic N pool. The latter is substantially greater than the plant-available (mineral) N fraction in the soil (McNeill and Unkovich 2007), and is made available slowly through mineralization.

Effects of grazing by large herbivores on ecosystem structure and processes have been described in a wide range of environments (Bowns and Bagley 1986; van de Koppel et al. 1997; van der Wal and Brooker 2004). At Hol, effects of grazing have been reported on plant abundance and traits (Austrheim et al. 2008b; Evju et al. 2006). The shrubs *Juniperus communis* and *Betula nana* increased at low sheep densities vs. non-grazed sites after four years of grazing whereas three species of graminoids increased at the high grazing treatment (Austrheim et al. 2008b). Moreover, at the plant community level Austrheim et al. (2008b) found reduced vascular plant height and plant cover after four years of grazing. Also, the percent of bare soil decreased less at high sheep density as compared to non grazed sites (Austrheim et al. 2008b). Together, these findings clearly indicate effects of sheep grazing on this system. However, as we found only small (indirect) effects of grazing on N-cycling, we cannot discern whether observed or potential future changes in ecosystem structure and processes are driven by indirect effects on N-cycling (Wardle et al. 2004) or direct effects on aboveground vegetation.

Conclusions

Based on N-content of four different plant species (all being protected from grazing) and $\delta^{15}N$ of seven different soil and plant components, we found little evidence for grazing induced effects on N-cycling. However, based on added ^{15}N-tracer recoveries and enrichments, there was some indication of greater N-cycling at grazed vs. non grazed sites. We conclude that grazing at the levels provided in this system, has only minor effects on N-cycling. Direct effects of grazing on aboveground vegetation may have greater impacts on ecosystem structure than indirect effects on N-cycling.

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