Norwegian University of Life Sciences
Faculty of Environmental Sciences
and Natural Resource Management

Philosophiae Doctor (PhD)
Thesis 2023:7

Subsoil nitrogen utilization and N_2O formation in Norwegian grass-clover forage systems

Nitrogenutnyttelse i dype jordlag og N_2O-dannelse fra kløvergras i norsk grovfôrproduksjon

Erin Elizabeth Byers

Subsoil nitrogen utilization and N_2O formation in Norwegian grass-clover forage systems

Nitrogenutnyttelse i dype jordlag og N_2O-dannelse fra kløvergras i norsk grovfôrproduksjon

Philosophiae Doctor (PhD) Thesis

Erin Elizabeth Byers

Norwegian University of Life Sciences
The PhD programme in Environmental Sciences
at the
Faculty of Environmental Sciences and Natural Resource Management

Ås 2023

Thesis number 2023:7
ISSN 1894-6402
ISBN 978-82-575-2035-9

Supervisors and Evaluation Committee

PhD SUPERVISORS
Professor Peter Dörsch, Main supervisor
Associate Professor Marina Azzaroli Bleken, Second supervisor
Associate Professor Susanne Eich-Greatorex, Additional supervisor

Faculty of Environmental Sciences and Natural Resource Management (MINA)
Norwegian University of Life Sciences
P.O. Box 5003 NMBU
1432 Ås
Norway

PhD EVALUATION COMMITTEE
Dr. Nyncke J. Hoekstra
Louis Bolk Institute
Kosterijland 3-5
3981 AJ Bunnik
The Netherlands

Dr. Reiner Ruser
University of Hohenheim
Schloss Hohenheim 1
70599 Stuttgart
Germany

COORDINATOR OF THE COMMITTEE
Dr. Vegard Martinsen
Faculty of Environmental Sciences and Natural Resource Management (MINA)
Norwegian University of Life Sciences
P.O. Box 5003 NMBU
1432 Ås
Norway

Acknowledgements

This PhD Thesis is submitted to the Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences (NMBU). I gratefully acknowledge Kunnskapsdepartementets funding which was granted by the Faculty at NMBU for the study period, additional funding from the Norwegian Research Council project AGROPRO (NFR 225330/E40), and in the final phase from my employer, the Norwegian Institute of Bioeconomy Research (NIBIO).

I am grateful for the leadership of Peter Dörsch, the main supervisor of this PhD, who balanced generosity in sharing knowledge with providing a basis for one to master a topic oneself. I am also grateful for Marina Bleken, who was the second supervisor and with whom I worked most extensively on writing, for an utmost commitment to quality research. Susanne Eich-Greatorex, the third supervisor of this PhD, provided a grounding and practical perspective to our ambitious work.

The extensive field and lab work of these studies would not have been possible without the help of Trygve Fredriksen, Lars Molstad, Øyvind Peder Vartdal, Toril Trædal, Prashanta Raut, Shimelis Gizachew Raji, Ragnhild Vold Karlsen, Sebastian Patzelt, and Fredrik Nerol Beilegaard. This work drew on the agronomic, biogeochemical, and field study capabilities of NMBU's Soil Group, the microbiological expertise of the NMBU Nitrogen Group, and its engineering capabilities supported by Lars Molstad. We utilized in-house isotope ratio mass spectrometry (IRMS) and gas chromatography (GC) capabilities, and a semiautonomous field robot for N_2O flux measurements developed by the NMBU Nitrogen Group and Adigo AS. I also thank Jan Vermaat, who provided academic feedback and support through challenges, and Lars Bakken, who gave profound inspiration and encouragement to pursue research. Finally, I thank Mirian Wangen, Brage Monsen and Kari Thue for support with the process of submitting the PhD.

I express deep gratitude for my family and friends, many of whom this journey has taken me far away from but who nonetheless have given their unwavering support through these years. To my parents, Rebecca Bryant and William Byers, whose unconditional love and encouragement give me strength to pursue the unknown. A list can never be complete, but I am especially grateful for my sister Rosemary Helvey-Byers, and to Briahn Martin, Cora McCold, Piper Mullins, Ben Fox-McCord, Natalie McLaurin, and Matthew Ball in the U.S., and Sunniva Fines, Béatrice Helgheim and Åsmund Kvifte in Norway. A special thank you to Stein Olav Kolle for his support in my completing this journey, and for assuring life continued as usual for our two-year old daughter, Astrid, while I was writing at all hours, and to his parents, Anne and Torfinn Kolle.

Table of Contents

Abbreviations and definitions

Biological nitrogen fixation (BNF): fixation of atmospheric N_2 to plant-available NH_4^+, in this context by rhizobia in association with roots of legumes such as clovers

Hemiboreal: a climate type which is between temperate and boreal

Subsoil: in this context, the highly compacted soil below the densest root zone (0-15 cm depth) and ploughed layer (down to 20 cm depth)

Nitrogen use efficiency (NUE): a term with many definitions which describe how completely nitrogen inputs are converted to outputs (for example forage yields); can also be applied to microbial and soil systems, and may also describe residence time and loss minimization

Diversity effect: a measurable change in outcome (for example increased forage yields) as a result of growing more than one species together (mixture), relative to the outcome when grown alone (pure stand) under the same conditions

Overyielding: a diversity effect observed as higher yields in a mixture than might be expected from its component species, based on their outcomes when grown in pure stands

Transgressive overyielding: Overyielding which results in a mixture yielding higher than the highest-yielding pure stand

Vertical niche differentiation: A diversity effect observed as changed rooting

N_2O Flux: The transient rate per area of nitrous oxide gas emissions from the soil surface to the atmosphere

List of papers

PAPER I

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina Azzaroli Bleken. Deep N acquisition in hemiboreal cultivated grasslands: I. Species in pure stands. Manuscript.

PAPER II

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina Azzaroli Bleken. Deep N acquisition in hemiboreal cultivated grasslands: II. Niche and overyielding effects in mixtures. Manuscript.

PAPER III

Erin Byers, Marina Azzaroli Bleken, Peter Dörsch, 2021. Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH. *Environmental Research Communications* 3, 015001, doi: 10.1088/2515-7620/abd623.

Abstract

Value creation in Norwegian agriculture is primarily based on milk and meat production, which depend in part on perennial grassland-based forage production. Roughly 60% of the fully cultivated land in Norway is used for grass production because the climate and growing conditions limit agronomic options for growing food-quality cereals and vegetables in many parts of the country. In addition, Norway has large areas of land which can be exploited as managed pasture or seasonal rangeland.

Globally, there is much focus on improving Nitrogen Use Efficiency (NUE) of crop production to mitigate the perturbation of the global nitrogen (N) cycle accompanying increased food production, with nitrate runoff leading to eutrophication of waterways, and emission of the climate-forcing gas nitrous oxide (N_2O). Perennial grasslands located in cold and northern climates are especially vulnerable to large N losses due to poor winter survival, long dormant periods, and decomposition of frost-killed biomass.

Much of Norway's cultivable land lies in the hemiboreal climate zone, to which many perennial grassland species are adapted. Perennial ryegrass (*Lolium perenne* L.), which is grown for its good yield potential but is less winter-hardy, can increase the risk of N losses if it survives poorly. Clovers, which are N-rich and more frost-sensitive than grasses, also contribute to N losses, especially to winter-associated N_2O production. On the other hand, clovers can increase the NUE of forage swards by partially replacing the need for fertilizer, and via diversity effects with grasses which increase sward yields and protein concentration.

Opportunities for improving NUE lie belowground. The dense mat of roots in the topsoil of grasslands cycles and stores massive amounts of N (and carbon and other nutrients) and is the locus for microbiological N transformations which also form N_2O. Some grassland species are capable of sending roots far below the densest root zone and recapturing N which has leached downwards. Diversity effects, not only between clovers and grasses, but also between grass species, greatly influence how

a grassland sward utilizes the N throughout the soil profile and throughout the growing season.

Using a stable isotope method with a novel slow-release $^{15}NH_4^+$ label, we studied deep root N uptake in well-established perennial forage swards. We studied five grass and two clover species in pure stands, over two growing seasons and a variety of weather events **(Paper I)**, as well as the diversity effects, or results arising from species interactions, on yields and deep N utilization by two grass-clover mixtures in the second growing season **(Paper II)**.

Tall-growing grass species proved effective at acquiring N from below the densest root zone in the mid- to late growing season, after apparently needing time to reestablish deep root activity in spring **(Paper I)**. The affinity for NH_4^+, the winter hardiness, and the growth vigor of these species proved to be more important functional traits for deep N acquisition than purported root depth **(Paper I)**. When in mixture, the importance of growth vigor "competitiveness" became even more important, stimulating changes in deep N uptake behavior between species **(Paper II)**. Clovers contributed to higher forage yields wherein grasses had higher N content, and mixtures utilized deep N as well as grass pure stands, thus diversity effects led to the best combination of yields and NUE **(Paper II)**.

In an adjacent field, we monitored N_2O formation in grass, clover, and grass-clover swards throughout winter, including prolonged reducing soil conditions under snowpack, and during spring thaw **(Paper III)**. We explored how liming, hypothesized to reduce N_2O formation by denitrification, affected N_2O emissions under these conditions *in situ*. Use of a fast-chamber robot allowed us to measure N_2O fluxes during thaw events at a high frequency, while we used pre-installed soil air probes and gas chromatography to monitor gas levels in subnivean soil air as indicators for microbiological N-cycling.

Off-season N_2O emissions were lowest in grasses, highest in red clover, and moderate in grass-clover mixtures, which emitted less than expected **(Paper III)**. Although liming reduced subsoil N_2O accumulation under snowpack in grass-only swards, we think that in clover-containing swards higher pH stimulated nitrification of N released by frost-killed clover biomass to NO_3^-, in turn stimulating N_2O production by nitrification or by providing substrate for denitrification. The apparent diversity effect wherein grass-clover mixtures emitted less N_2O than

expected was observed in both limed and non-limed plots in autumn. However, this effect was weaker in limed mixtures in the spring, suggesting increased N cycling in the higher-pH soils became more important than decomposition of clover biomass to N_2O production as the next growing season began.

This thesis demonstrates synergistic diversity effects of combining clover with grasses, which results in reduced N losses combined with increased protein yields, and possibly reducing the severity of N_2O formation due to clovers over winter. NUE and N_2O emission in Norwegian forage production can be managed by careful choice of forage species, particularly considering the proportions of clover and appropriate pH management.

Norsk sammendrag

Verdiskaping i det Norske landbruket baserer seg hovedsakelig på produksjon av melk og kjøtt, og er delvis avhengig av fôrproduksjon basert på flerårig eng. Cirka 60% av fulldyrket areal i Norge brukes til flerårig grasvekst som er velegnet også i deler av landet hvor klima og vekstforhold begrenser korn- og grønnsaksproduksjon. I tillegg har Norge store områder med areal som kan utnyttes som gjødslet innmarksbeite eller utmarksbeite.

Globalt er det stor fokus på å forbedre nitrogeneffektiviteten (nitrogen use efficiency; NUE) i planteproduksjon for å motvirke forstyrrelsen av den globale nitrogensyklusen som følger med økt matproduksjon. Nitratavrenning fører til eutrofiering av vann og vassdrag, og N bruk i matproduksjon øker utslipp av klimagassen lystgass (N_2O). Flerårig eng som finnes i kalde og nordlige områder er spesielt sårbare for nitrogen-tap på grunn av dårlig overvintring, lange perioder uten vekst, og nedbrytning av frostdrept biomasse.

Mye av Norges fulldyrkede areal ligger i klimasonen «hemiboreal», hvor mange flerårige grasarter er tilpasset et kaldt vinterklima gjennom vinterherding. Flerårig raigras (*Lolium perenne* L.), som dyrkes på grunn av sitt gode avlingspotensial men som er mindre vinterhardt, kan øke sjansen for N tap hvis det overlever dårlig. Kløver, som er N-rik og som tåler frost dårligere enn gras, bidrar særlig til N-tap, spesielt om vinteren i form av lystgass, men på den andres siden kan kløver øke NUE i fôrproduksjonen ved å delvis erstatte tilført gjødselmengden. Sammen med gras bidrar kløver til diversitetseffekter som øker fôravlingene og proteinmengde.

Muligheter for å øke NUE ligger under bakken. Den tette matten av røtter til grasvekster i øvre matjordlaget lagrer og sirkulerer store mengder N (samt karbon og andre næringsstoffer). I dette området omsetter mikroorganismer C og N og danner N_2O underveis. Noen arter kan ha røttene langt ned i jorden og fanger opp N som har blitt vasket ned i jordprofilen. Diversitetseffekter, ikke bare mellom kløver og gras, men også mellom ulike grasarter, påvirker i stor grad N-utnyttelsen i hele jordprofilen, og gjennom sesongen.

Vi kombinerte en stabil isotopmetode med en unik langvarig $^{15}NH_4^+$ merking for å undersøke N-opptak av dypgående røtter i veletablerte flerårige eng. Vi undersøkte fem gras- og to kløverarter i monokultur, i to vekstsesonger og gjennom flere ulike værhendelser **(Artikkel I)**. Vi undersøkte diversitetseffekter, eller resultater som oppstår fra interaksjoner mellom plantearter, på avlinger og dyp N-utnyttelse i to blandinger av gras og kløver (kløvergras) i den andre vekstsesongen **(Artikkel II)**.

De høyvoksende grasartene utnyttet N fra dyp jord effektivt fra midten til slutten av vekstsesongen, men trengte tid til å gjenopprette dyp rot aktivitet om våren **(Artikkel I)**. Affinitet for NH_4^+ opptak, grad av vinterherding og vekststyrke av disse artene viste seg å være viktigere funksjonelle egenskaper for dyp N-utnyttelse enn tidligere antatt rotdybde **(Artikkel I)**. I kløvergras blandinger ble vekststyrke eller konkurranseevne enda viktigere, og stimulerte artene til å endre dyp N-utnyttelse på ulike måter **(Artikkel II)**. Kløveren bidro til økt fôravling og høyere N-innhold i gras. Kløvergras blandinger utnyttet også dyp N like godt som grasmonokultur. Derfor førte diversitetseffekter til den beste kombinasjonen av avling, kvalitet og NUE **(Artikkel II)**.

I et tilgrensede felt undersøkte vi N_2O produksjon i gras-, kløver-, og kløvergraseng gjennom en vinter, som inkluderte en periode med langvarig reduserende jordforhold under snødekke, og i løpet av tiningsperioden om våren **(Artikkel III)**. Vi undersøkte hvordan kalking, som er antatt å redusere N_2O-dannelse fra denitrifikasjon, påvirket N_2O-utslipp under slike forhold *in situ*. Vi målte N_2O-fluks med høy frekvens under fryse-tinehendelser ved bruk av en robot utstyrt med hurtigkamre («fast-box» chambers). Vi brukte forhåndsinstallerte jordluftsonder og gasskromatografi for å undersøke gassnivåer i jordluftet gjennom vinteren, som brukes som indikatorer for mikrobiologisk N-prosesser.

Utslipp av N_2O om vinteren var minst i gras, størst i rødkløver, og moderat i kløvergras, hvor utslippet var lavere enn forventet **(Artikkel III)**. Mens kalking reduserte N_2O i jordluften under snødekke i rene grasruter, resultatene indikerer at den høyere pH kan ha stimulert nitrifikasjon etter nedbrytning av N-rik biomasse fra frostskadet kløver, og at dette førte til økt N_2O-produksjon ved nitrifikasjon eller ved å gi substrat for økt denitrifikasjon. Den tydelige diversitetseffekter der kløvergras ga mindre N_2O utslipp enn forventet ble observert både i kalket og ikke-kalket vekster om høsten, men effekten var svakere i kalket jord om våren. Det kan

betyr at økt N-sirkulering ved høyere pH ble en viktigere kilde for N_2O enn nedbrytningen av kløverbiomasse ved start av vekstsesongen.

Denne avhandlingen dokumenterer noen synergiske diversitetseffekter av å blande kløver med gras i grovfôrproduksjon, som resulterer i redusert N-tap kombinert med økt proteinavling, og muligens med en redusert grad av N_2O-utslipp utenom vekstsesongen. NUE og N_2O-utslipp i norske fôrproduksjon kan påvirkes ved nøye sammensetning av fôrarter, spesielt med tanke på andel kløver i vekst, og hensiktsmessig pH-behandling.

Synopsis

1 Introduction

Human activity at present more than doubles the amount of nitrogen (N) transferred from stable N_2 in the atmosphere to reactive forms of N in terrestrial pools, largely through use of synthetic fertilizers and cultivation of legumes in food production (Fowler et al., 2013). Soil mineral nitrogen, including nitrate (NO_3^-) and ammonium (NH_4^+), is a main growth-limiting factor for plants and is the primary ingredient in mineral fertilizer, followed by phosphorus and potassium. The more water-soluble nitrate is often lost from agricultural fields via runoff and leaching and can lead to eutrophication of waterways. Cultivated fields are also a primary source of the climate-forcing gas nitrous oxide (N_2O), an intermediary of microbial N transformations in soil, stimulated by N addition and other disturbances which encourage N mineralization (Dalal et al., 2003; Tian et al., 2019). N_2O contributes approximately 4% in CO_2-equivalents of the annual anthropogenic greenhouse gas emission to the atmosphere (IPCC, 2022), where it also contributes to ozone depletion (Ravishankara et al., 2009).

In 2020, Norwegian agriculture accounted for around 29,000 tons (metric) of total N to water (50% more than from Norway's wastewater, land-based industry and landfills combined; Guerrero and Sample, 2022) and 6,000 tons of N_2O to air (77% of the country's anthropogenic N_2O emissions; Miljødirektoratet, 2022). Only the Norwegian aquaculture industry is a larger source of N pollution, releasing nearly 70,000 tons of N to water in 2020.

1.1 A need for improved forage systems

Agricultural activity in Norway is centered around forage crops grown for meat and milk production. Norwegian-grown forage consists of perennial grasses and some cereals, mainly well-adapted spring barley, and also oats, and wheat when cold growing conditions prevent it from reaching bread quality (Koga et al., 2016). Together they contribute 52%, and grazing another 7%, to the nutritional demands of the national animal production industry; the remainder is satisfied by imported feed concentrates (Landbruksdirektoratet, 2021). Only a little over 8,000 km^2, or

about 2.5% of mainland Norway is fully cultivated, of which 60% is used for grass forage and 35% for grains; additionally 1,800 km^2 of surface-cultivable land (overflatedyrket jord) and on-farm pasture land (innmarksbeite) is managed as grassland (SSB, 2022).

There are many questions beyond the scope of this thesis about the sustainability and food security of the Norwegian agricultural system. Improving grassland yields and increasing protein content could in theory reduce the necessity of importing feed concentrates for cattle and sheep, providing some buffer from geopolitical uncertainty, and from what is seen as less sustainable agricultural production in parts of the world from which raw ingredients for feed concentrates are sourced (Cadillo-Benalcazar et al., 2020). However, high-concentrate diets have led to increased milk yields per cow (Landrø Hjelt et al., 2019; Volden, 2019), and concentrate feed can be cheaper than grassland-based forage (Thuen and Tufte, 2019), challenging this goal.

In connection with the Paris Agreement, Norway has committed to reducing greenhouse gas emissions. The government does not expect the agricultural sector to eliminate emissions as early or to the extent of other sectors, as the first priority is producing food (Klima- og miljødepartementet, 2021). Efforts will be a collaboration between the country's agricultural organizations and the government, which has committed to promote dietary recommendations and reduction of food waste, and to continue to facilitate the industry in making climate-smart agricultural choices.

The goal of increasing grassland forage quality implies increasing the overall N content in plant herbage and thus in the agroecosystem, increasing both the quantity of N inputs and the potential for N losses. This seems at odds with the goal of reducing agriculture's impacts on the climate and environment. The present thesis focuses on how underground N retention, cycling and transformations in the plant-soil system of Norway's hemiboreal forage grasslands can reconcile these divergent goals.

1.2 Nitrogen use efficiency in grasslands

Perennial grasslands, with a dense mat of roots and organic matter near the soil surface which stores and cycles nutrients, and plants which store N late-season for

regrowth early the following spring, are generally thought to have a high nitrogen use efficiency (NUE) in terms of yields per applied mineral fertilizer. NUE in cultivated grasslands should be seen as a cumulative average over their several-year lifecycle which ends in ploughing (Bleken et al., 2022). In-season NUE can for a while surpass 100% at fertilization rates below 50 kg N ha^{-1} yr^{-1} by mining the large N pool stored in the top soil layer and from biological nitrogen fixation (BNF), though optimal yields may occur with closer to 200 kg N ha^{-1} yr^{-1}, and NUE closer to 65% (Höglind et al., 2020). Realized NUE during the growing seasons depends on pairing the timing of fertilization to plant regrowth in the early spring and after each of multiple harvests per year (de Boer et al., 2016).

NUE can be expressed in several ways beyond yields per applied mineral fertilizer (sometimes called fertilizer use efficiency). Mean residence time (MRT) of N refers to the length of time N is retained in plant biomass and available for use before it is lost (Berendse and Aerts, 1987). A higher MRT implies better NUE. Losses occur continuously by litter shedding, herbivory by grazers and also smaller animals and soil organisms, root exudation, and N loss from leaves (Bowatte et al., 2014). In forage grass species selected to yield strongly with high N inputs, the MRT tends to be lower compared to species adapted to low-N environments (Vazquez De Aldana and Berendse, 1997). MRT was inversely correlated to fertilizer use efficiency (here designated A), in that species adapted to low-N conditions had high MRT and low A, and species adapted to high-N conditions had low MRT and high A, but when the latter were grown in low-N conditions, their yields per N input (A) were reduced.

This is to say that there are physiological constraints to selecting both for high forage yields and reduced N losses. Efficient uptake and reuptake of N which is in the soil profile can compensate for a low MRT, helping to close this gap. Perennial grassland species differ in their ability to recover N from deeper soil, and it is thought that combining species with different rooting depths can increase utilization of N throughout the soil (Hoekstra et al., 2015; Hooper, 1998; Jumpponen et al., 2002; Pirhofer-Walzl et al., 2013). This diversity effect is called vertical niche differentiation if different species respond to being grown in the mixture by changing their root uptake behavior to better exploit different soil depths for nutrients (Husse et al., 2017; Mommer et al., 2010). There is ongoing research on the conditions under which the functional trait of deep N uptake is expressed, because root presence does not necessarily indicate active uptake by those roots. Further background on these issues is given in **Papers I and II**.

Most Norwegian leys are sown as mixtures based on timothy (*Phleum pratense* L.), meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.), red and white clover (*Trifolium pratense* L. and *Trifolium repens* L.), and perennial ryegrass (*Lolium perenne* L.), which is relatively newer to Norwegian use (Ergon et al., 2016). Also of interest in Norway is tall fescue (*Schedonorus arundinaceus* Schreb.), which is high yielding and drought resistant but has lower forage quality than meadow fescue. Perennial ryegrass and tall fescue are thought of as complementary and can be grown together, or the ryegrass-fescue hybrid *Festulolium* can be used (Cougnon et al., 2014). Tall fescue has deep roots and has been used in a few N uptake studies as a treatment assumed *a priori* to have superior uptake from depth (Hernandez and Picon-Cochard, 2016; Malcolm et al., 2015).

NUE in terms of yields per applied mineral fertilizer can also be improved by growing clovers with grasses. Clovers require less N fertilizer due to BNF, and their presence can increase the N concentration of grasses while high clover N content is maintained (Nyfeler et al., 2011). The improved nutritional status of grasses can in turn lead to the diversity effect overyielding, or higher yields in a mixture than could be expected from its component species when grown in pure stands. If mixture yields surpass the highest-yielding pure stand, overyielding is called transgressive (Schmid et al., 2008). Several diversity effects may lead to overyielding, as mentioned in **Paper II**, and herbage overyielding combined with increased total N content of the herbage is a very desirable agronomic outcome. Clovers are not a loss-free N source, however, and contribute to N leaching and N_2O formation, particularly outside of the growing season (Sturite et al., 2021). The large influence N_2O has on climate forcing outweighs its tiny contribution (Smith et al., 2012) to the quantity of N lost, and it is not easily described by the NUE definitions above.

1.3 Challenges in a hemiboreal climate

Under the Köppen climate classification system, areas along the southern edge of Norway and to either side of the Oslo fjord, and a region along the arctic circle which is warmed by the Gulf Stream, are considered to be hemiboreal ("Dfb", Kottek et al., 2006), or halfway between a temperate (European) and boreal climate (most of the rest of Norway). In contrast to the climate of much of Western Europe, which is classified as temperate oceanic ("Cfb"), the hemiboreal climate type is characterized by colder winters, with the coldest month having an average temperature of -3 C° or

below. Due to the high latitude, Norwegian agricultural is characterized by very low solar radiation in winter, and high radiation and long daylength in summer.
For perennial grassland plants to survive harsh winters, they must cease growth and make adaptations to the photosynthetic apparatus before it is damaged by low temperatures (Sandve et al., 2011). Species such as perennial ryegrass and the ryegrass-fescue hybrid *Festulolium* are non-native to northern climates and do not respond as well to cues of light and temperature to cease growth, particularly in northern and inland Norway (Østrem et al., 2015b). This problem could increasingly affect more southern areas of Norway as climate scenarios predict warmer and cloudier autumns (Dalmannsdóttir, 2015). However, these species have a higher growth potential than fescues when they survive well, and are the subject of breeding efforts for winter hardiness (Østrem et al., 2015a). Winter-adaptive improvements have been made to the legumes alfalfa (*Medicago sativa* L.) and red and white clover, although red clover remains a challenging legume species to grow in cold-winter climates (Abberton and Marshall, 2005; Annicchiarico et al., 2015). In order to contribute to N uptake and reuptake, plants must survive and persist **(Paper I)**, and diversity effects can in turn affect survival and persistence (Ergon et al., 2016, **Paper II**).

Clover is more N-rich and frost sensitive compared to grasses, leading to N losses during freeze-thaw. Frost-damaged plants release labile biomass containing readily bioavailable carbon and nitrogen, which trigger flushes of microbiological activity including nitrification and denitrification. N_2O is a byproduct of nitrification, the microbial oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-). This process is mediated by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and requires oxygen (Prosser et al., 2020). N_2O is an obligatory intermediate in denitrification, a facultative anaerobic respiration process which oxidizes C (or other reduced compounds) to reduce N from NO_3^- to N_2 via NO_2^-, nitric oxide (NO), and N_2O. The final reduction step to N_2 is catalyzed by the enzyme N_2O reductase. Denitrification enzymes are induced by anoxia, but production of functioning N_2O reductase takes time (Bakken et al., 2012). This may be one reason for bursts of N_2O emissions after precipitation followed by dry weather (wetting-drying), or after brief saturation with meltwater from freeze-thaw, because aerobic conditions return before N_2O is fully converted to N_2.

Half or more of the annual N_2O emissions from agricultural soils which undergo freeze-thaw occur off-season, comprising 17-28% of agriculture's N_2O emissions

globally (Wagner-Riddle et al., 2017). Grassland sites in the temperate oceanic climate show higher variability of N_2O emissions than annual croplands (Rees et al., 2013). In the hemiboreal climate, agricultural soils have shown larger peak N_2O fluxes and larger interannual variability in N_2O emissions than in temperate oceanic Europe (Freibauer and Kaltschmitt, 2003). This may be in part due to clovers which contribute disproportionately to winter emissions in climates with harsh winters. Even defoliation of white clover in late autumn was found not to reduce winter N_2O emissions, but rather reduced the swards' ability to store N overwinter and take N up again in the spring (Sturite et al., 2021, 2007, 2006).

Although denitrification is thought of as the main source of freeze-thaw related N_2O from agriculture (Congreves et al., 2018; Mørkved et al., 2006; Risk et al., 2013), nitrification must occur at a significant rate to provide NO_3^- as a substrate for denitrification. A flush of labile C and NH_4^+ nitrified to NO_3^- might in turn fuel denitrification at rates which overwhelm available N_2O reductase. This is important because liming has been proposed to mitigate denitrification-derived N_2O by creating a pH more favorable to maturation of N_2O reductase (Bakken et al., 2012; Kunhikrishnan et al., 2016; Liu et al., 2014), but this may not help in situations with high rates of nitrification. Liming also greatly increases nitrification rates (Parton et al., 2001) and shifts the balance from AOA to AOB, which produce more N_2O (Nadeem et al., 2020). Liming has been shown nonetheless to halve off-season N_2O emissions from hemiboreal autumn-ploughed grasslands leys; this held true in soils where grasses, clovers, and grass-clover mixtures had been grown (Bleken and Rittl, 2022).

1.4 Research aims

This body of research focuses on how species composition affects yields and belowground N processes in perennial forage swards grown in the Norwegian hemiboreal climate. **Paper I** assesses different species in pure stands for their ability to take up N present in deeper soil below the densest root zone (such as leached N), improving NUE by recirculating N to plants during the growing season. **Paper II** explores how combining grasses and clovers in mixtures can lead to diversity effects improving the uptake of deep N, along with diversity effects on herbage yields and N concentration, improving forage production. **Paper III** explores the problem that including clover in forage swards increases N_2O

formation outside of the growing season, and evaluates whether clover proportion and liming can mitigate this effect.

Our approach was based on *in situ* ^{15}N labeling of deep soil and subsequent analysis of ^{15}N in bulk soil and herbage by isotope ratio mass spectrometry (IRMS, **Papers I and II**), N_2O fluxes estimated by the fast-chamber technique, and subsoil N_2O accumulation measured by gas chromatography analysis of soil air samples **(Paper III)**. Ancillary soil moisture and temperature data were collected by dataloggers in winter **(Paper III)**, and KCl-extraction for soil mineral N was performed at the beginning and end of the growing seasons **(Papers I-III)**. In both experiments we greatly emphasized weather in our interpretation of specific time periods in the data.

Our research objectives were:

1. To test a novel slow-release ^{15}N-labeling method as to its suitability for studying deep N uptake by forage plants over a prolonged period, overcoming a common limitation that ^{15}N label disperses quickly in soils **(Paper I)**
2. To investigate how well perennial grass and clover species with different purported rooting depths take up N from below the densest root zone in a hemiboreal Norwegian grassland **(Papers I and II)**
3. To examine how growing conditions, such as seasonality and weather events, as well as species persistence, influence deep N uptake by forage species **(Papers I and II)**
4. To explore how plant species diversity influences deep N uptake in forage mixtures, both due to intraspecies competition and synergistic improvement of N concentration in grasses due to N transfer from clovers **(Paper II)**
5. To investigate the extent of and processes by which N_2O is formed in grass-clover mixtures during winter, and the possible mitigative effects of liming **(Paper III)**
6. To add to the knowledge about NUE and reducing N losses at the end of the growing season, as connected to species choice in hemiboreal forage production **(Papers I-III)**

2 Growing season and winter conditions

Field experiments were performed in two adjacent experimental fields located in Ås, Norway at approximately 59°39'47"N 10°45'39"E, and 70 m above sea level. Criteria for the hemiboreal climate classification include the warmest months having a mean temperature below 22°C and the coldest month below -3°C (Kottek et al., 2006). During our study period, the summer months had average temperatures between 14-16°C, and the winters had at least one month with an average temperature below -3°C, except for the winter of 2016-2017 which did not have as long or deep cold snaps, and the lowest monthly average temperature was -1.9°C in February (Wolff et al., 2018). Nonetheless, the sites underwent repeated freeze-thaw each winter, stressing the clovers and perennial ryegrass. White clover barely survived to the spring of 2016. Ryegrass overwintered poorly each year, especially to the spring of 2017, though it recovered by mid-season each year **(Papers I and II)**. In the summer of 2017 before the second harvest, a prolonged drought affected all treatments in the ^{15}N labeling experiment **(Papers I and II)**. The 2017-2018 winter was especially cold and had continuous snow and ice cover from January to April; ryegrass, red clover and white clover all overwintered poorly to spring of 2018 **(Papers II and III)**.

The research fields consisted of a stone-free silty loam artificially drained at 1 m depth, a usual practice given the typically waterlogged hydrology of Norway's arable soils. We chose 42 cm as the depth of interest for placing a ^{15}N label to test plants' ability to recover N from below the densest root zone **(Papers I and II)**. The densest root zone in our swards was at 0-15 cm depth. In another study on one of our fields only 4-6% of the total root biomass from 0-30 cm was found in the naturally compact subsoil below 23 cm (Bleken et al., 2022). In the ploughed zone down to 20 cm depth, bulk density is 1.15 g cm^{-2}, but the subsoil is highly compacted and bulk density increases markedly to 1.5 g cm^{-2} at 40 cm depth **(Paper I)**. To investigate accumulation of N_2O in the soil profile under snow and ice cover, we chose 8, 24 and 40 cm depth, to represent the densest root zone, the subsoil, and the threshold just below the ploughed zone where the soil becomes denser **(Paper III)**.

3 Methods and findings: Deep N uptake (Root study)

3.1 Root study: Experimental design

Root uptake studies often apply tracers to the soil and estimate uptake by measuring tracer presence in plant biomass, commonly using N compounds (NH_4^+, NO_3^-, urea) enriched with the stable isotope ^{15}N, though other tracers (Hoekstra et al., 2014a) can be used, for example cesium, strontium and lithium (Mamolos et al., 1995), or ^{18}O (Hoekstra et al., 2014b). Because N compounds cycle rapidly between inorganic and organic N pools in soil, a common limitation is that the short-lived ^{15}N label must be tracked within days or weeks before it dissipates evenly among all pools of interest or is lost, for example as leached NO_3^-.

The research aims of studying how weather events, persistence, growth vigor, and plant diversity influence deep N uptake, required a study period long enough to capture phenomena which take more time to develop than a typical ^{15}N labeling campaign would cover. We therefore adsorbed 98 AT% $^{15}NH_4^+$ to clinoptilolite, a natural zeolite which adsorbs and desorbs NH_4^+ via ion exchange with pseudo-second-order kinetics (Milovanović et al., 2015), and placed it at our depth of interest (42 cm) using a 4 x 4 grid of 16 holes spaced 12 cm apart and set in between plant rows. We harvested herbage from an area wider than the labeling area to assure near-complete recovery of label which might be transported horizontally outward as well as upward by plant roots ("negative discard" method, (Kristensen and Thorup-Kristensen, 2007; Powlson and Barraclough, 1993). All the harvested herbage from each experimental subplot was dried, chopped, ground, and well-mixed before a representative sample was ball-milled to a fine powder and analyzed by IRMS for ^{15}N enrichment. With this method we could estimate the actual mg of ^{15}N taken up into the herbage in relation to the mg of ^{15}N placed in each subplot.

Paper I evaluates the method's success. In some treatments we accounted for 85-90% of the applied ^{15}N label after two years, in herbage from the three harvests per year plus soil sampled after the sixth harvest. Additionally, 2% or less was found in

herbage taken from a 10 cm margin just outside our sampling area, indicating our negative-discard method captured most of the ^{15}N label moved horizontally by plant activity.

Our investigation of deep N uptake was split into two papers due to the complexity of the experiment. **Paper I** presents two ^{15}N labeling campaigns, in 2016 and 2017, in pure stands of four commonly-grown grasses as discussed in the introduction of this Synopsis: **perennial ryegrass, tall fescue, meadow fescue,** and **timothy**, hereafter referred to as "tall-growing grasses", plus a low-growing, shallow-rooted **Kentucky bluegrass** (*Poa pratensis* L.) and **red and white clover**. Our hypothesized order of ^{15}N uptake ability was based on *a priori* assumptions that the deepest-rooting plants (tall fescue, red clover) would recover more deep N than shallower-rooting plants (perennial ryegrass, bluegrass, white clover), and that clovers would recover less ^{15}N than the grasses because BNF contributes to their total N acquisition, reducing their need for deep-sourced N.

> Hypothesized order from most to least recovered ^{15}N: Tall fescue, timothy and meadow fescue, perennial ryegrass, bluegrass, red clover, white clover.

We expected to see stronger ^{15}N recovery correspond to stronger herbage yields (dry matter, designated DM). For example, we expected weaker ^{15}N recovery by winter-damaged ryegrass in spring, and weaker ^{15}N recovery by all species in autumn when yields were lower than in spring or summer. We also explored whether N deficiency could explain ^{15}N recovery strength.

Paper II presents the same experiment carried out on two mixtures in 2017. The first, **Mix 4**, contained **perennial ryegrass, timothy, tall fescue** and **red clover**, thus blending tall-growing grasses which were presumed deep-rooting (tall fescue), shallow-rooting (ryegrass), and widely-used (timothy), with a presumed deep-rooting legume. The second mixture, **Mix 10**, included the species of Mix 4, plus **meadow fescue**, which is also commonly used, presumed shallow-rooting **Kentucky bluegrass** and **white clover**, plus three species which were not part of the ^{15}N labeling campaigns in pure stands and thus not analyzed in depth (*Festulolium, Medicago sativa* L., and *Bromus inermis* Leyss.). The seven species presented in **Paper I** were analyzed for diversity effects when grown in mixtures in **Paper II**, by comparing their performance to that in pure stands. Additionally, we

assessed overyielding of DM and N yields, as well as "overyielding" of deep N uptake by the mixtures.
In all cases, the experiment was carried out on subplots in a fully-randomized block grassland trial. Labeling campaigns were carried out on four replicate subplots of each treatment. The subplots labeled in 2016 were harvested for two growing seasons (three cuts per year), and the subplots labeled in 2017 were harvested for one growing season plus the subsequent spring harvest.

3.2 Root study: Analytical approach

The analytical approach compensated for interrelated phenomena which distorted the results. All of the equations used are printed in **Papers I and II**, and a summary of the variables used for analysis is presented in

Table 1 of this Synopsis.

Firstly, because we tracked uptake of a known amount of label (68 mg ^{15}N m^{-2}) over multiple harvests, any subsequent harvests after the first one must account for the ^{15}N remaining in each subplot after removal of herbage in previous harvests, in order to compare treatments on a like basis in later harvests. In **Paper I**, the mg ^{15}N harvested in herbage divided by the mg ^{15}N remaining in each subplot, is denoted *^{15}N recovery strength*.

Secondly, species in pure stand differed in DM yields and N concentrations (i.e. bluegrass generally has a higher N concentration than tall-growing grasses, and N-fixing clovers have the highest N concentrations). We therefore found it useful for differentiating the species' deep N uptake behavior to define two new variables which weighted ^{15}N recovery strength by DM (*Recovery per Dry Matter*, "RDM"), and by N (*Relative Deep Uptake Index*, "RDUI"). The name *relative deep uptake index* indicates that it describes the relative contribution of deep-sourced (^{15}N-enriched) N relative to a plant's total N acquisition.

To analyze whether N deficiency was related to ^{15}N uptake in pure stands, we had to account for the fact that plants become more fibrous and decrease in N concentration as they grow. Each plant has a critical level of N which allows full expression of growth potential at any given time depending on the growth stage reached. Comparing raw N concentrations (N per DM) directly between treatments

can therefore lead to misinterpretation. We estimated each pure stand's N status at the time of harvest as a function of N concentration and DM following equations given by Baadshaug and Lantinga (2002). We were not able to do this for species grown in mixture because the estimation is based on DM per area, to which mixture component species contribute only in part. However, in **Paper II** we found it useful to analyze the difference in N concentration of a species grown in mixture compared to pure stands.

Table 1. Overview of variables used to compare treatments in pure stands, mixtures as a whole, and component species in the mixtures

Paper I	Realized yields:			Derived variables:			
Pure stands	g DM m^{-2}	g N m^{-2}	mg ^{15}N m^{-2}	^{15}N recovery strength *	N status	RDM *	RDUI *
Paper II	Realized yields, can be compared directly to pure stands:			Overyielding (realized – expected yields):			
Mixtures as a whole	g DM m^{-2}	g N m^{-2}	mg ^{15}N m^{-2}	Δ g DM m^{-2}	Δ g N m^{-2}	Δ mg ^{15}N m^{-2} *	
	"Behavior" diversity effects which can be compared directly to pure stands:			"Outcome" of diversity effects on yields, which sum up to mixture overyielding, but cannot be compared directly to pure stands:			
Component species in mixtures	Δ N conc. (N/DM)	Δ RDM *	Δ RDUI *	Δ g DM m^{-2}	Δ g N m^{-2}	Δ mg ^{15}N m^{-2} *	

(*) – indicates that differences in remaining ^{15}N were accounted for
(Δ) – indicates the difference between the realized value in mixture and the expected value based on performance in pure stands

Paper II compares yields of mixtures as a whole to those of pure stands to determine if overyielding and/or transgressive overyielding occurred. The mixture species proportions had changed since sowing in 2014 (seed was sown in equal proportions by weight), so we calculated expected DM, N, and ^{15}N yields as the share of DM each species occupied in the mixture, multiplied by its performance in pure stands. We treated the overyielding of DM, N, and ^{15}N uptake separately; for example, the expected mg ^{15}N uptake was based only on ^{15}N uptake by the pure stands, independent of whether the species was overyielding in N or DM. The three types of overyielding (DM, N, and deep N uptake) must all be considered in context,

because improved N acquisition inherently affects photosynthesis and thus root growth.

In **Paper II**, we were also interested in how the behavior of the individual component species differed in mixtures compared to their performance in pure stands. We focused on the change in N concentration (N/DM), and the changes in RDM and RDUI (^{15}N/DM and ^{15}N/N respectively, adjusted for differences in ^{15}N remaining in the subplots).

In an attempt to find relationships between the variables, in **Paper I** we performed ANOVA on linear models of ^{15}N recovery strength as explained by species, N status and harvest. We also performed a Pearson correlation test between ^{15}N recovery strength and the variables DM yield, N yield, N concentration, N status, RDM and RDUI. This was done separately for all grass samples together, and all clover samples together. In **Paper II**, we placed individual observations of component species on scatter plots where the x and y axes were pairs of diversity effects. Of these, we selected five plots which best showed a differentiation in the species' changed behaviors when grown in mixtures.

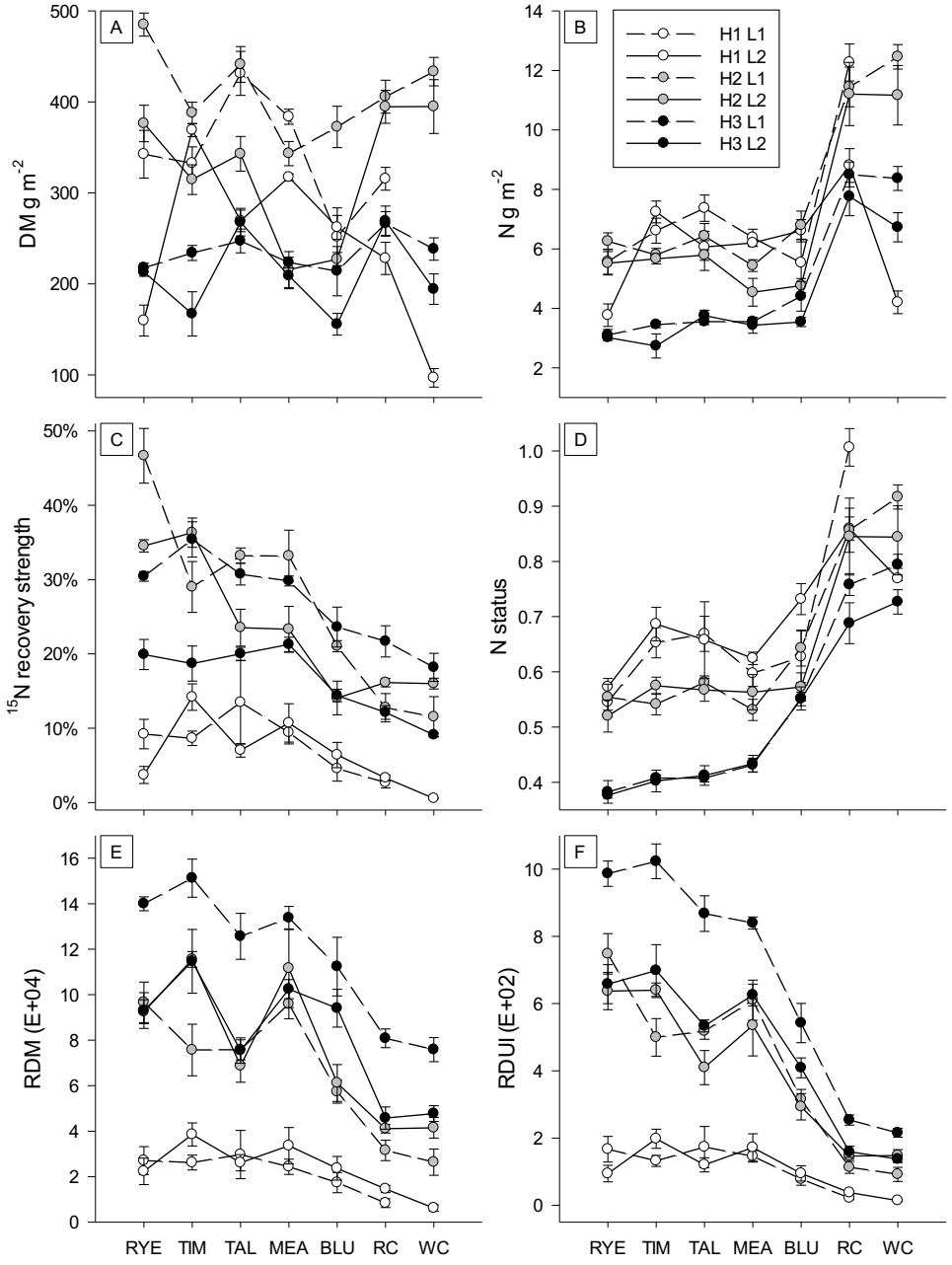


Figure 1: (A) **DM yield** (g DM m^{-2}), (B) **N yield** (g N m^{-2}) of harvests H1 through H3 after labeling L1 in 2016 and L2 in 2017. (C) **^{15}N recovery strength** (% recovered of label remaining in soil, Eqn. 3), (D) **N status** (1 indicates no deficiency), (E) **RDM** (^{15}N recovery strength per DM), and (F) **RDUI** (^{15}N recovery strength per N), Mean values of 4 replicates (±SE). A line connects results within each harvest for readability. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover (not harvested in spring of L1).

3.3 Root study: Findings in pure stands

All treatments recovered little ^{15}N in the spring (Figure 1 C). We did not foresee this, as DM yields were similarly high in spring and summer (Figure 1 A). It seems to indicate that deep root activity takes time to re-establish after overwintering, both for grasses, which form new tillers, and for clovers including red clover, which despite its taproot may also increase its depth of root uptake activity throughout the season. ^{15}N recovery strength was equally high in autumn as in summer despite lower DM yields in autumn (Figure 1 A, C). This was also surprising, and considering the fact that N status was lowest in autumn (Figure 1 D), may indicate that N deficiency increased deep N uptake in autumn.

There was not enough variation in N status within single species at single harvests to find explanatory power on ^{15}N recovery strength using ANOVA; rather, the seasonal effect (first, second, or third harvest) was more significant. To further explore whether N deficiency can stimulate deep N uptake, we would have to design an experiment manipulating N deficiency *ceteris paribus*, perhaps separately in spring, summer and autumn.

RDUI and RDM clearly distinguish the species in Figure 1 E and F. Our hypothesized order from greatest to least ability to recover ^{15}N from depth was: tall fescue, timothy and meadow fescue, ryegrass, bluegrass, red clover, and white clover. Tall fescue did not show superior ^{15}N uptake in pure stands despite good yields, not even in the drought-affected summer of 2017 despite its demonstrated drought tolerance. The other tall-growing grasses, including purportedly shallow-rooting perennial ryegrass, were equally capable of $^{15}NH_4^+$ uptake from 42 cm depth as tall fescue. Bluegrass performed in between the tall-growing grasses and the clovers, as expected. Red and white clover had similar RDM and RDUI, showing that red clover's deep taproot structure did not confer advantageous deep N uptake.

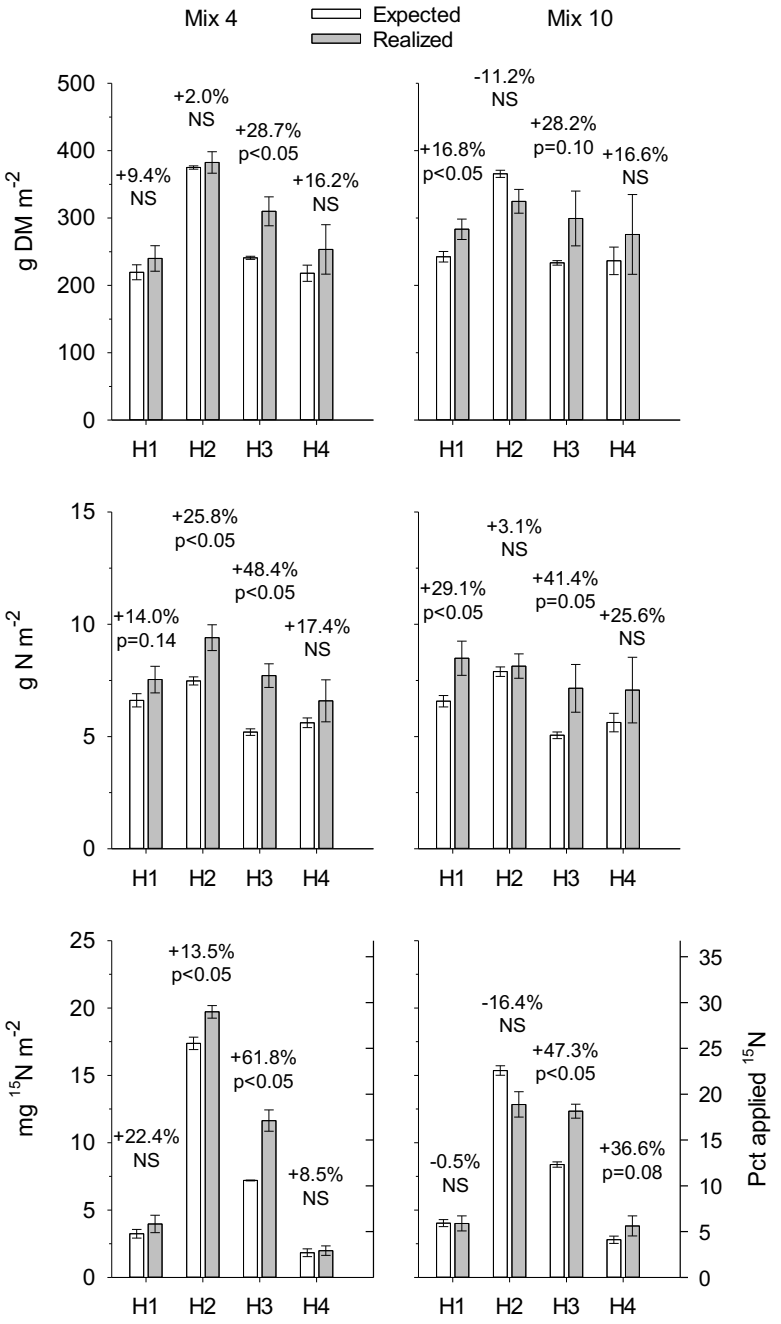


Figure 2: Overyielding in mixtures in four consecutive harvests (H1 to H4) of: **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}), and total **^{15}N herbage uptake** (mg ^{15}N m^{-2}). Mean values of 4 replicates (±SE). Percent change and significance of one-sided t-test for positive overyielding is shown above bars. NS: p>0.15.

3.4 Root study: Findings in mixtures

The two grass-clover mixtures overyielded in DM, N and ^{15}N recovery. Mixtures' yields were more evenly distributed throughout the season than those of pure stands, and had ^{15}N recovery similar to that by tall-growing grass pure stands and N yields similar to those in clover pure stands. Taken together, mixtures showed an overall advantage over pure stands. Though neither DM, N, or ^{15}N overyielding was transgressive for the whole growing season, ^{15}N overyielding in the autumn harvest was higher than the best-recovering pure stand (meadow fescue), and was even more prominent than autumn N overyielding (Figure 2).

Grasses grown in the mixtures increased in N concentration compared to pure stands while clovers maintained their N concentration, indicating a possible stimulation of BNF which benefitted grasses as explored in Nyfeler et al. (2011). Tall fescue, meadow fescue and ryegrass nonetheless realized positive diversity effects on ^{15}N recovery (
Figure **3**), indicating that if clover inclusion did suppress deep N uptake in grasses, it did not outweigh other effects which increased deep N uptake. The increased N concentration in these species could have supported assimilation and root growth. Timothy, however, showed a negative diversity effect on ^{15}N uptake, which was surprising because it was among the highest-uptaking species in pure stand. Bluegrass and the clovers showed negative diversity effects on ^{15}N uptake, as expected.

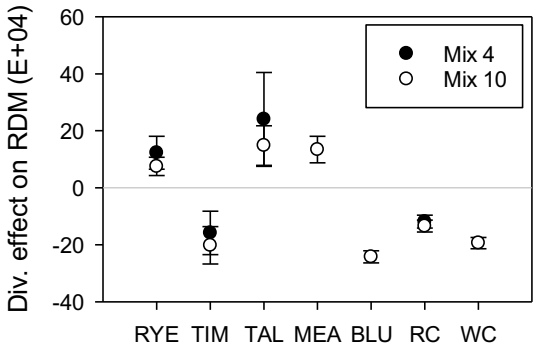


Figure 3: Cumulative diversity effects on RDM (realized - expected values): ^{15}N recovery per Dry Matter (mg ^{15}N g^{-1} DM), shown in each species in Mix 4 and Mix 10. Weighted average of four replicates for all four harvests taken, ±SE.

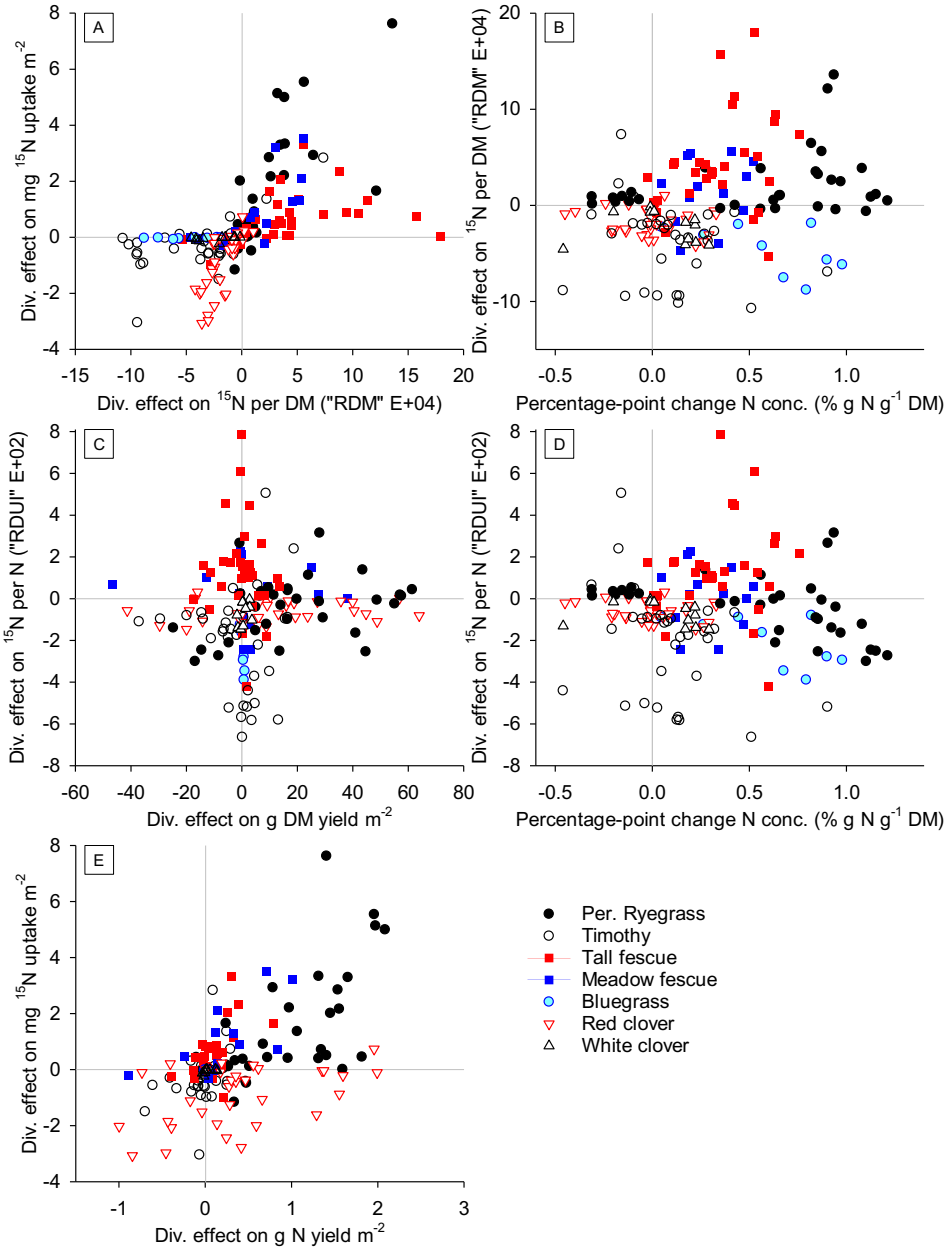


Figure 4: Relationships between selected diversity effects (realized - expected values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All observations are plotted from four replicates of each mixture at each of four harvests taken.

We selected five scatter plots to show interesting relationships between different diversity effects in individual species when grown in mixture. The species' positive or negative contributions (mg m^{-2}) to ^{15}N uptake overyielding by the mixture, when plotted against the diversity effect on RDM, show that changed deep N uptake patterns and not simply DM overyielding led to ^{15}N overyielding (Figure 4 A). Species varied in positive or negative changes to growth vigor (g DM m^{-2} versus expected), with only perennial ryegrass and red clover strongly increasing in growth vigor, which did not correlate with observed changes in RDUI. Conversely, tall fescue increased RDUI in the absence of changes to growth vigor (Figure 4 C).

Plotting the diversity effect on RDM versus the change in N concentration shows that perennial ryegrass and tall fescue (and to a lesser extent meadow fescue) were unique in increasing both in N concentration and deep N uptake (Figure 4 B). Of these, ryegrass contributed the most to overall sward N and ^{15}N due to its DM overyielding (Figure 4 E). Bluegrass increased its already-high N concentration but reduced its deep N uptake. Timothy had unchanged or decreased N concentration, and as mentioned, greatly reduced its RDM compared to pure stands, which were among the best at recovering deep N. The clovers also had varying changes to N concentration, but decreased deep N uptake less than did timothy or bluegrass (Figure 4 B).

A clear difference in behavior between tall fescue and ryegrass can be seen by comparing Figure 4 B to D, which is similar but shows RDUI instead of RDM. At high increases of N concentration (to the right along the x-axes), ryegrass showed a positive RDM together with a nil or negative RDUI, showing that in these instances, ryegrass increased N uptake from all soil depths but relatively more from non-deep sources, such as from the dense near-surface root zone or via N transfer from legumes. This possibly put competitive pressure on tall fescue, stimulating it to increase deep N uptake. This dynamic between tall fescue and ryegrass was mainly apparent in the drought-affected summer and N-deficient autumn harvests.

4 Methods and findings: off-season N_2O formation (Overwinter study)

4.1 Overwinter study: Experimental design

To study the contributions of clovers to off-season N_2O formation in soils with differing pH, we quantified the amount of N_2O formed throughout winter 2017-2018 in an adjacent-plot experiment of limed and non-limed grass, grass-clover, and clover swards. This allowed us to investigate whether liming, which is predicted to reduce N_2O emissions by supporting formation of N_2O reductase, can mitigate N_2O formation in clover or mixtures containing clover.

Plant treatments included (1) a **grass-only sward** containing timothy, perennial ryegrass, meadow fescue, tall fescue, and Kentucky bluegrass; (2) a **grass-clover mixture** containing the grass species above plus red clover, and (3) a **pure stand of red clover**. Eight replicate subplots of each treatment were selected from within a fully-randomized block trial. Of the eight replicates per plant treatment, four had been **limed** with 23 t ha^{-1} dolomite in 2014 (pH_{CaCl2} 6.09 in December 2017), and four were untreated **control** plots (pH 5.18). The larger experiment in which we placed our study subplots had varying levels of fertilization. We chose to use grass-only plots fertilized at a high level (270 kg N ha^{-1} yr^{-1}), grass-clover plots fertilized at a moderate level (140 kg N ha^{-1} yr^{-1}), and red clover pure stands receiving no fertilizer. We did this in order to increase the clarity of contrasts we expected to find: i.e. any N_2O formed in clover pure stands could not be attributed to mineral fertilizer, and we expected the lower level of fertilization in grass-clover mixtures to stimulate BNF by the red clover (See **Paper II**).

Gases produced in soil travel via diffusive exchange with the atmosphere, though equilibrium is never reached; soil air always has more CO_2 and a bit less O_2 than air above the surface. Bursts of produced N_2O temporarily reside in soil air before the gas is emitted from the soil surface and can be measured, for example by flux chambers. Under snow and ice cover, gases including N_2O diffuse more slowly and can accumulate in the soil profile (Burton and Beauchamp, 1994). We therefore

measured both N_2O surface fluxes from all replicates, and sampled soil air from 8, 24 and 40 cm depths in three of the four replicates per treatment throughout the winter. We tracked the depth of the soil freezing front using three dataloggers placed throughout the field, equipped with volumetric water content and temperature sensors placed at 0 cm (in the stubble), 5, 24, and 40 cm depths. We also installed three removable "frost tubes" in the soil, filled with water and blue dye (McCool and Molnau, 1984). Details are given in **Paper III**.

Fluxes of N_2O from soil to atmosphere have large spatiotemporal variability; microbial activity produces N_2O in "hot spots" flush with N substrate, NH_4^+ for nitrification or NO_3^- for denitrification, and at "hot moments" of hypoxia/anoxia (Bakken and Frostegård, 2020; Li et al., 2015). A major challenge with measuring N_2O fluxes from the soil surface using chamber methods is that chambers can only be placed for a limited time and must then be removed so that the soil-plant system remains undisturbed. Manual chamber methods are time-consuming and often locked into the position of a preinstalled frame. We opted for a fast-chamber technique, utilizing a semiautonomous robot which navigated a predetermined route to perform flux measurements with 120-second chamber deployment time (compared to 30-60 minutes with manual chambers). This allowed us to measure the subplots up to three times per day during peak N_2O flux events during the snow-free period. Due to the tight spacing of subplots, we measured fluxes from the exact same spot every time, though the robot is capable of varying the measurement position.

4.2 Overwinter study: Analytical approach

In addition to looking at N_2O flux and soil air gas concentration dynamics throughout winter, we calculated cumulative emissions and subsoil gas accumulation in different time periods: the freeze-thaw impacted autumn, a period of deep snow cover, the period of spring thaw, and the first days of the growing season after thawing was complete and spring fertilizer had been applied (Figure 5). Cumulative fluxes were calculated by interpolating between individual measurements multiplied by the amount of time between them. We did not interpolate between 12 December and 6 April, when it was not possible to drive the flux robot in deep snow. For soil air, we calculated a time-integrated N_2O amount per known soil volume with units g N_2O-N m^{-3} * days, expressing both the concentration and length of time that concentration was present in soil. We used

ANOVA with *post hoc* Tukey tests to determine statistical difference between treatments' N_2O accumulation and emission, which are shown for each time period in Figure 5. We considered these results alongside ancillary weather and soil freezing conditions in order to surmise the microbiological processes involved.

4.3 Overwinter study: Findings

Grass-only swards, which received the most fertilizer, yielded significantly more than the unfertilized red clover pure stands at the last harvest before the overwinter experiment began, with grass-clover mixtures yielding in between. Limed red clover pure stands yielded more DM than their control; otherwise limed and non-limed plots yielded the same for grass-only, and grass-clover mixtures respectively. Even with lower yields and no fertilizer, red clover pure stands emitted by far the most N_2O fluxes during freeze-thaw and early spring thaw and accumulated larger amounts of N_2O in the soil profile during autumn and winter than grass-only plots (Figure 5). Grass-clover mixtures emitted and accumulated significantly more N_2O than grass-only swards and significantly less than clover pure stands. Only after thawing was complete and spring fertilizer was applied at the end of April did grass-only plots show the highest N_2O fluxes (soil air probes were removed before spring fertilization).

In the autumn freeze-thaw period, limed red clover pure stands emitted more N_2O than their control, contrary to the supposed mitigative effect of liming. As introduced in this Synopsis (Challenges in a hemiboreal climate), large quantities of frost-killed C- and N-rich clover tissues could have made nitrification the limiting factor for N_2O production, rather than denitrification as normally assumed. The pH of the limed plots was more favorable for nitrification than that of the control plots, and a faster nitrification rate could overwhelm the denitrifiers' capacity to reduce N_2O to N_2, despite liming having increased the amount of functional N_2O reductase. Thus, in addition to nitrification being itself a source for N_2O, it could indirectly increase N_2O by supplying substrate for denitrification. An alternate explanation could be that the limed red clover pure stands had higher autumn yields than the non-limed ones, thus returning more C and N to the soil during winter. We did not see an accompanying increase in soil-accumulated N_2O in limed red clover pure stands during this time period, which may be due to the more reducing conditions deeper in the soil favoring denitrification.

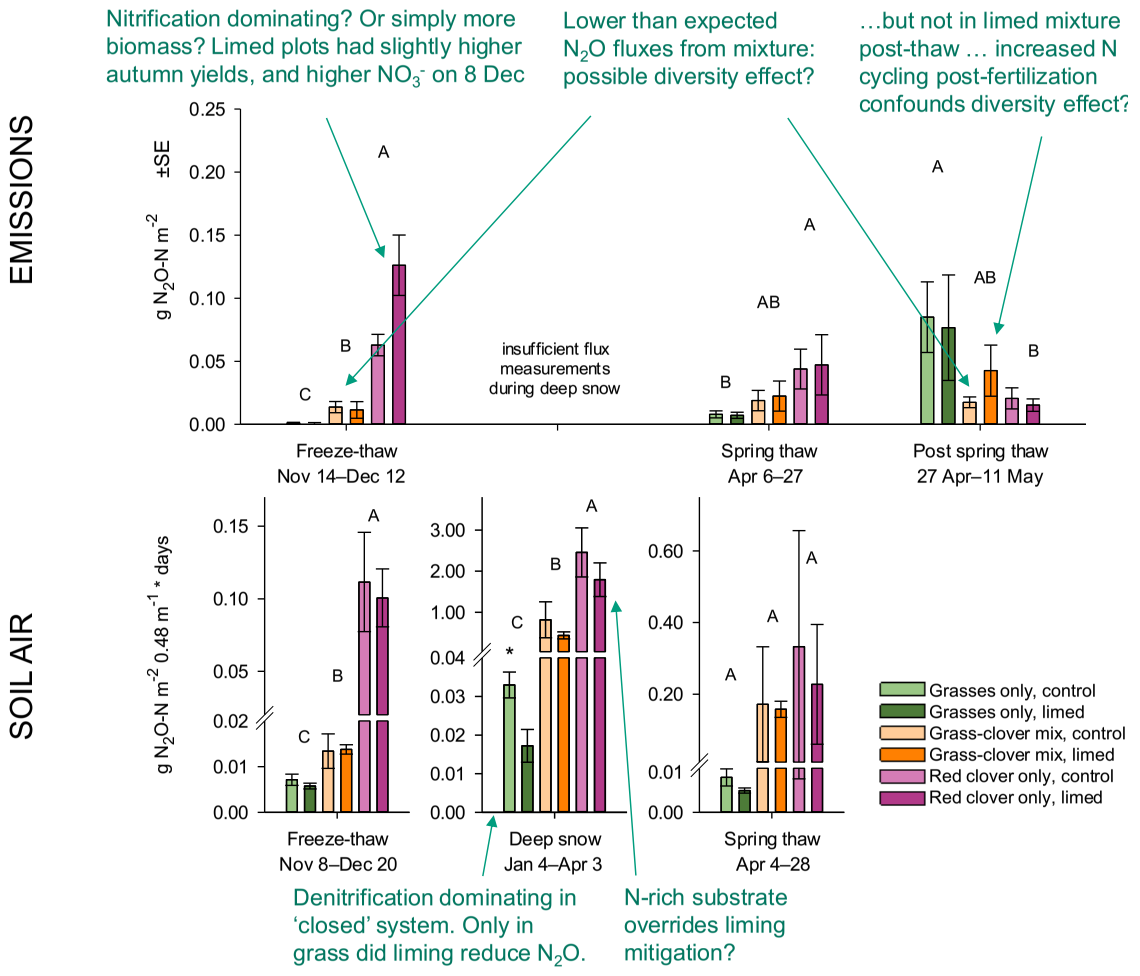


Figure 5: For each seasonal division (not equal in length by days):

(Top) Cumulative N_2O emissions interpolated from flux estimates (mean g N m^{-2} ±SE of 4 replicates)

(Bottom) Time-integrated N_2O accumulation in 0–48 cm soil depth (mean g N m^{-2} * 0.48 m^{-1} * days ±SE of 3 replicates

Letters indicate Tukey groupings ($p<0.05$) of species effect on the natural log of the measurement. (*) indicates the only significant mitigative liming effect found, on the natural log of the integrals of soil N_2O accumulation for grass-only plots under deep snow (one-sided t test, $p=0.05$).

We did see the expected mitigative effect of liming on N_2O production in one instance: N_2O accumulation was lower in the soil of limed grass-only swards than their control under deep snow. Ancillary GC data showed reduced O_2 and increased CO_2 during the two-month snow-covered period, indicating a semi-closed, reducing soil environment where anaerobic metabolism continued and denitrification likely dominated, with good conversion of N_2O to N_2. Grass-clover mixtures and red clover pure stands showed a tendency towards, but were not significantly lowered by liming. Similar to in autumn, this suggests that N_2O reduction capacity was overwhelmed by available substrate in the plots containing N-rich clover biomass.

Finally, there was some evidence of a diversity effect of growing grasses together with clovers on N_2O emissions in the freeze-thaw autumn period. A rough calculation, similar to the expected yield calculation in **Paper II,** was made based on the proportions of grass versus clover DM in each mixture subplot, multiplied by the emissions observed during the same time period in the grass-only or clover pure stand swards. In the freeze-thaw autumn period, the grass-clover mixtures emitted less than a third of the expected N_2O, both in the limed and non-limed control replicates. This mitigative grass-clover diversity effect was weakened in limed mixtures during spring thaw, and nearly disappeared after fertilization, while non-limed control mixtures continued to emit less N_2O than expected, suggesting increased N substrate in the limed mixture overwhelmed the diversity effect as well as the pH effect on N_2O reduction. This could be due to several factors. Grass-only swards became the leading N_2O emitters in spring, also in the week before fertilization, while pure clover swards had more bare soil and little herbage after ice receded, were slow to regreen, and reduced their N_2O emissions as spring progressed. This suggests mixtures had a different N source than frost-killed clover biomass, which may have been used up by spring. It also suggests that liming, which is expected to increase N mineralization and thus nitrification, may have increased N cycling such that a possible diversity effect was obscured. Similar to the limed pure red clover stands emitting more N_2O in autumn than the non-limed red clover, nitrification in the grass-clover mixture may have become a more important N_2O source than denitrification in spring.

We did not attempt to calculate a diversity effect on over-winter N_2O accumulation in the soil air, but Figure 5 suggests there was an autumnal diversity effect reducing soil air N_2O accumulation under the grass-clover mixtures, corresponding to their lower-than-expected autumnal N_2O fluxes. Under deep snow, there was no apparent

grass-clover diversity effect on soil air N_2O accumulation. The weekly measurements (Figure 6) indicate a reducing environment when diffusive exchange with the atmosphere was hindered by deep snow and ice, which favored more complete denitrification to N_2. An initially higher N_2O accumulation under the clover pure stands compared to the grass-clover mixture was evened out by mid-February, when some N_2O appears to have been consumed. Under deep snow, the liming effect was also visible in red clover pure stands and grass-clover mixture plots, but as mentioned this was not significant as it was in grass-only swards during deep snow (Figure 5).

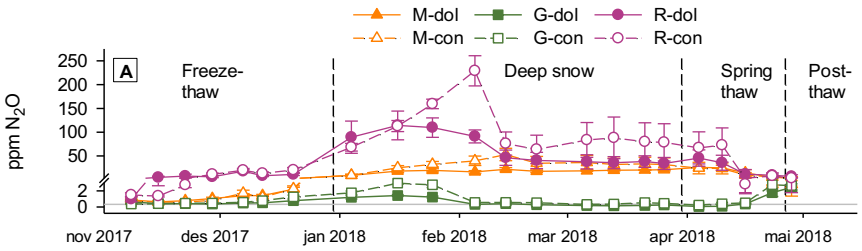


Figure 6: Concentration of N_2O in soil air for each treatment. M: grass-clover mixture; G: grass-only sward, R: red clover pure stand; dol: limed; con: control. Mean (±SE) of 3 replicates at 3 depths (n=9). Gray lines indicate the ambient atmospheric level of N_2O (0.32 ppm).

5 Common discussion

5.1 Methods

The slow-release ^{15}N label method **(Papers I and II)** enabled performing a root uptake study over a time period sufficient to capture seasonal development of deep-rooting behavior by different species, influences of weather events and growing conditions, species persistence over winter, and diversity effects on these factors due to interactions between species. The field robot equipped with a fast-chamber N_2O analyzer **(Paper III)** allowed multiple N_2O flux measurements per day and (although not utilized by us) can measure at any point along the ground surface, addressing the challenge of high spatiotemporal variability of N_2O formation and emission. These methods can facilitate investigation of NUE in grasslands in cold-winter climates, which have a propensity for high and highly variable peak N_2O emissions driven by freeze-thaw, and which sustain large N losses from clovers. This can support plant breeding (Abberton and Marshall, 2005; Annicchiarico et al., 2015; Østrem et al., 2015a) and seed mixture development which is focused on adapting plants to both the existing cold-climate challenges and expected increases in temperature, precipitation and cloud cover (affecting photoacclimation).

5.2 Managing for NUE in hemiboreal grasslands

Papers I and II address improving NUE by optimizing the uptake and reuptake of N from throughout the soil profile, including N which has been transported below the densest root zone. In pure stands **(Paper I)**, the well-adapted grasses timothy and meadow fescue, as well as ryegrass (as long as it rebounds from winter), were equally capable as tall fescue of acquiring NH_4^+ from ~40 cm depth. This is to say that all of them are a good choice for this function and should not be excluded from consideration on the presumption that they will have inferior deep N uptake to tall fescue. **Paper I** warns that we cannot expect any of the species tested to recover deep N in the spring, as it appears deep root activity takes time to reestablish after overwintering. However, deep N recovery was better than expected in the autumn, when plants become N deficient and low yielding. This may help mitigate autumn NO_3^- leaching and freeze-thaw related N_2O emission.

Paper II shows that grasses benefited in N concentration by being grown in mixture with clovers (improving NUE in terms of forage protein per N input), and that this did not prevent observable overall increases in deep N uptake by the mixtures (improving NUE via reducing losses). Mixtures overyielded in ^{15}N recovery to such an extent in autumn that they surpassed the ^{15}N recovery of any pure stand, this despite containing low-uptaking clovers! The species which drove the improved deep N uptake most in mixtures were perennial ryegrass and tall fescue. Perennial ryegrass yielded more vigorously and increased its N concentration enormously in mixtures, while maintaining strong deep N uptake as in pure stands, suggesting ryegrass was very competitive for N from non-deep sources. Tall fescue did not increase growth vigor in mixtures, and showed only moderate increases in N concentration, but competition from ryegrass may have stimulated tall fescue to increase its deep N uptake.

Tall fescue demonstrated its superior ability for deep N uptake when exposed to diversity effects in mixtures, especially later in the growing season **(Paper II)**. Meadow fescue showed diversity effects in between those of ryegrass and tall fescue. While we observed that timothy's contribution to deep N uptake in mixtures was diminished by its apparent out-competition, given timothy's excellent deep N uptake in pure stand, no evidence suggested any of these tall-growing grasses should be excluded from consideration on the basis of poor deep N utilization.

Shallow-spreading, stolon-forming white clover was surprisingly as capable of deep N uptake as red clover, which has a persistent taproot **(Paper I)**, though white clover had poorer early spring yields and was outcompeted in mixtures **(Paper II)**. Red clover is known as a non-persistent species which declines three or so years after seeding in forage swards. The more persistent white clover, which is also tolerant to trampling and grazing, is often used in permanent pastures and may be important to deep N utilization in those systems. Similarly, bluegrass, which is low-growing and less competitive for light in swards with red clover and tall-growing grasses, showed moderate ability to recover deep N in pure stands and may also be important to deep N utilization in pastures **(Papers I and II)**. While forage swards are often ploughed and renewed by the time red clover declines, there is a practice of "frost seeding" red clover into degraded permanent pasture in autumn, when it can establish without strong competition from grasses (Annicchiarico et al., 2015; Leep, 1989). This technique is also adapted to cover crops, for example as frost-

seeding red clover between rows of wheat (Gaudin et al., 2013). All of the species we tested had little deep N uptake in spring after overwintering **(Paper I)**; this does not suggest an immediate benefit in deep N uptake by frost-seeded clovers, but such a technique could reintroduce legumes and their benefits in subsequent years of longer-term or more permanent swards.

Paper III addresses extra N_2O formed off-season by inclusion of (red) clover in mixtures with grasses, a negative climate outcome which frustrates the advantages clover inclusion gives for increasing NUE. In testing whether liming mitigated clover- and freeze-thaw associated N_2O formation, we found that mineralization of N-rich clover substrates in the absence of plant N uptake appears to increase nitrification activity, an effect that is only enhanced by liming to raise soil pH above 6.0 (Parton et al., 2001).

There was however evidence of a diversity effect in the grass-clover mixtures which reduced the N_2O emissions relative to what one might expect based on the proportion of clover in the sward. This held true for both limed and non-limed subplots during autumn freeze-thaw, and in the control during spring thaw, but liming weakened this diversity effect in spring, again suggesting nitrification was dominating. The fact that grass-only swards surpassed pure red clover in N_2O emissions as the soil warmed suggests frost-killed clover biomass was no longer the N source leading to N_2O emissions in the limed grass-clover mixture in spring, but rather an increased rate of N mineralization due to liming.

5.3 Clover proportion

The proportion of (predominantly red) clover in the root study was around 40% by herbage DM on average in the third production year **(Paper II)**, and in the overwinter study was 38% in the limed subplots and 49% in the control, in the last harvest of the second production year just before the experiment **(Paper III)**.

Modeling by Fuchs et al. (2020) demonstrated that partially replacing fertilizer with legumes in temperate grasslands can reduce N_2O emissions while maintaining productivity, but note that the prior level of fertilization and the amount being replaced, as well as climate and the potential for freeze-thaw cycles, affect the outcome locally. In-season rain or drought spells can also affect the outcome; Hansen et al. (2014) in Western Norway found that drying-rewetting associated N_2O

emission *in a drought* year was positively correlated with clover proportion in swards, while no such relationship was found in a non-drought year. Fuchs et al. (2020) concluded that to minimize N_2O, the best proportion of clovers is 30-50%, with a fertilizer level no higher than 150 kg N ha^{-1} yr^{-1}. This fertilization level is somewhat lower than the 200 kg N ha^{-1} yr^{-1} proposed by the model of Höglind et al. (2020) for optimal yields in Scandinavia. The grass-clover mixture plots in our overwinter study received 140 kg N ha^{-1} yr^{-1} **(Paper III)**.

Optimal N overyielding and N transfer from clovers to grasses, improving NUE and forage quality, was found by Nyfeler et al. (2011) to occur with between 40-60% clover by DM at a site near Zurich, Switzerland receiving 150 kg N ha^{-1} yr^{-1}. A local study in Ås receiving 100 kg N ha^{-1} yr^{-1} noted that white clover should be sown at a higher seeding rate than red clover to maximize N overyielding benefits, due to red clover's high canopy outcompeting other species for light capture, but that some red clover was probably necessary (Ergon et al., 2016).

5.4 Ploughing: the end of the NUE cycle

Ploughing for sward renewal or rotation is a major source of N losses in grasslands which was not included in this research work. Reinsch et al. (2018) showed that high soil moisture in autumn and subsequent freeze-thaw were major causes of N_2O emissions in the year after ploughing a grassland in northern Germany. On the same field used in the overwinter study in this work **(Paper III)**, a ploughing study was performed as part of a different project one year later, using the same fast-chamber robot to measure N_2O (Bleken and Rittl, 2022). The study found that liming halved, on average, cumulative N_2O emissions measured during the off-season from ploughing in September 2018 until May 2019. The same treatments were used as in **Paper III:** unfertilized red clover pure stands showed a weaker mitigative effect on N_2O by liming, with limed plots emitting 70% of the amount of N_2O compared to their non-limed control; grass-clover mixtures receiving 140 kg N ha^{-1} yr^{-1} and grass-only swards receiving 270 kg N ha^{-1} yr^{-1} showed a stronger liming effect, emitting just 32% of the emissions compared to their control plots. Interestingly, the ploughing study also included grass-clover mixtures receiving the higher fertilizer dose (270 kg N ha^{-1} yr^{-1}), and their liming effect was the same as in red clover pure stands (70% of the emissions from their control).

Table 2 summarizes the average N_2O fluxes in each treatment in the overwinter study versus the ploughing study for autumn versus spring. Though the dates do not align well, they comprise similar freeze-thaw and snowmelt conditions (2017-2018 was a particularly harsh winter with long snow cover). Whereas living red clover pure stands emitted by far the most N_2O in both autumn and spring (Byers et al., 2021), after ploughing, the grass-clover mix emitted the most N_2O, perhaps reflecting its systematic application of mineral N fertilizer (140 kg N ha^{-1} yr^{-1}) compared to the unfertilized red clover. The high-fertilized (270 kg N ha^{-1} yr^{-1}) grass-clover mixtures in the ploughing study emitted even more N_2O (Bleken and Rittl, 2022). Perhaps the diversity effect reducing freeze-thaw N_2O emissions below what was expected from grass-clover mixtures (Overwinter study: Findings) involves increased stored N in the plant-soil system, which is released upon ploughing. This implies a trade-off between N retention during a sward's lifetime and N loss upon grassland renewal.

Table 2. Average N_2O emissions (µg N m^{-2} h^{-1}) in different time periods of the studies on **Live** crops overwinter (Byers et al., 2021), and **Ploughed** plots (Bleken and Rittl, 2022) one year later, on the same field in Ås. Each column is labeled with the name as given and date range in the respective study. Cumulative fluxes were not calculated in Bleken and Rittl (2022); average flux data from the Live study (Byers et al., 2021) was not published in the article but was derived from raw data. The highest-emitting limed and control treatment are bolded in each time period.

	Live	Ploughed		Live	Ploughed	
Fertilization Grass-only: **270** kg N ha^{-1} yr^{-1} Red clover: **0** kg N ha^{-1} yr Grass-clover: **140** kg N ha^{-1} yr^{-1} **Treatment**	**Freeze-thaw 14 Nov – 12 Dec, 2017**	**Freeze-thaw 23 Oct – 11 Dec, 2018**	**Snow 11 Dec – 23 Jan, 2018**	**Spring thaw 6 Apr – 27 Apr, 2018**	**Melting 26 Feb – 25 Mar, 2019**	**Spring 25 mar – 2 Apr, 2019**
Grass-only, limed	1	60	19	11	70	**28.7**
Grass-only, control	2	144	44	13	419	49.5
Red clover pure stands, limed	**178**	75	**78**	**76**	85	7.2
Red clover pure stands, control	**93**	151	26	**69**	160	32.1
Grass-clover mix, limed	16	**118**	51	49	**159**	17.5
Grass-clover mix, control	20	**328**	**207**	40	**729**	**81.5**

5.5 Final remarks

Temporary grasslands which are fertilized and ploughed as part of a crop rotation perturb the plant-soil system and create opportunities for microbial N cycling and N loss at several stages. This thesis demonstrates that NUE can be improved in-season by careful choice of forage species, taking advantage of diversity effects between grasses of different rooting depths and competitive strategies, and synergistic N transfer from clovers to grasses. The work also demonstrates that off-season N_2O emission can be managed first and foremost by avoiding high proportions of clover, and by appropriate pH management.

The long use of grassland resources for human calories though hunting, herding, husbandry and forage production may one day soon include direct protein extraction for human consumption (Du et al., 2020; Nørgaard et al., 2018), addressing NUE on a trophic level. Solutions may also address the rotation system, such as sowing "catch crops" to utilize excess N in the soil immediately after ploughing a grassland (Hansen and Eriksen, 2016). Despite the mixed results on the mitigative effect of liming on off-season N_2O emissions **(Paper III)**, the fact that liming halved the winter N_2O emissions after ploughing limed soils, for all sward compositions (Bleken and Rittl, 2022), stresses that pH management is agronomically vital and a valid strategy for N_2O mitigation.

Reinsch et al. (2018) suggest delaying grassland ploughing until spring, when soil is drier, or to opt for permanent grasslands. Our findings in **Papers I and II** that deep N recovery was better than expected leading up to autumn, especially in grass-clover mixtures, and our findings in **Paper III** that grass-clover mixtures may also show a diversity effect reducing autumnal freeze-thaw N_2O emissions in living swards compared to their clover proportion, reinforce this view. That grass-clover mixtures and not pure red clover had the highest winter N_2O emissions after ploughing (Bleken and Rittl, 2022), however, implies a trade-off between N retention during a sward's lifetime and N loss upon grassland renewal.

6 References

Abberton, M.T., Marshall, A.H., 2005. Progress in breeding perennial clovers for temperate agriculture. J. Agric. Sci. 143, 117–135. https://doi.org/10.1017/S0021859605005101

Annicchiarico, P., Barrett, B., Brummer, E.C., Julier, B., Marshall, A.H., 2015. Achievements and Challenges in Improving Temperate Perennial Forage Legumes. Crit. Rev. Plant Sci. 34, 327–380. https://doi.org/10.1080/07352689.2014.898462

Baadshaug, O.H., Lantinga, E.A., 2002. ENGNOR, a Grassland Crop Growth Model for High Latitudes. Technical Documentation. (Reports from AUN. Department of Plant Sciences No. 2/2002). Agricultural University of Norway.

Bakken, L.R., Bergaust, L., Liu, B., Frostegård, Å., 2012. Regulation of denitrification at the cellular level: a clue to the understanding of N_2O emissions from soils. Philos. Trans. R. Soc. B Biol. Sci. 367, 1226–1234. https://doi.org/10.1098/rstb.2011.0321

Bakken, L.R., Frostegård, Å., 2020. Emerging options for mitigating N_2O emissions from food production by manipulating the soil microbiota. Curr. Opin. Environ. Sustain. 47, 89–94. https://doi.org/10.1016/j.cosust.2020.08.010

Berendse, F., Aerts, R., 1987. Nitrogen-Use-Efficiency: A Biologically Meaningful Definition? Funct. Ecol. 1, 293–296.

Bleken, M.A., Rittl, T.F., 2022. Soil pH-increase strongly mitigated N_2O emissions following ploughing of grass and clover swards in autumn: A winter field study. Sci. Total Environ. 828, 154059. https://doi.org/10.1016/j.scitotenv.2022.154059

Bleken, M.A., Rittl, T.F., Nadeem, S., Hansen, S., 2022. Roots and other residues from leys with or without red clover: Quality and effects on N_2O emission factors in a partly frozen soil following autumn ploughing. Sci. Total Environ. 831, 154582. https://doi.org/10.1016/j.scitotenv.2022.154582

Bowatte, S., Newton, P.C.D., Theobald, P., Brock, S., Hunt, C., Lieffering, M., Sevier, S., Gebbie, S., Luo, D., 2014. Emissions of nitrous oxide from the leaves of grasses. Plant Soil 374, 275–283. https://doi.org/10.1007/s11104-013-1879-6

Burton, D.L., Beauchamp, E.G., 1994. Profile Nitrous Oxide and Carbon Dioxide Concentrations in a Soil Subject to Freezing. Soil Sci. Soc. Am. J. 58, 115. https://doi.org/10.2136/sssaj1994.03615995005800010016x

Byers, E., Bleken, M.A., Dörsch, P., 2021. Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH. Environ. Res. Commun. 3, 015001. https://doi.org/10.1088/2515-7620/abd623

Cadillo-Benalcazar, J.J., Renner, A., Giampietro, M., 2020. A multiscale integrated analysis of the factors characterizing the sustainability of food systems in Europe. J. Environ. Manage. 271, 110944. https://doi.org/10.1016/j.jenvman.2020.110944

Congreves, K.A., Wagner-Riddle, C., Si, B.C., Clough, T.J., 2018. Nitrous oxide emissions and biogeochemical responses to soil freezing-thawing and drying-wetting. Soil Biol. Biochem. 117, 5–15. https://doi.org/10.1016/j.soilbio.2017.10.040

Cougnon, M., Baert, J., Van Waes, C., Reheul, D., 2014. Performance and quality of tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) and mixtures of both species grown with or without white clover (*Trifolium repens* L.) under cutting management. Grass Forage Sci. 69, 666–677. https://doi.org/10.1111/gfs.12102

Dalal, R.C., Wang, W., Robertson, G.P., Parton, W.J., 2003. Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. Soil Res. 41, 165. https://doi.org/10.1071/SR02064

Dalmannsdóttir, S., 2015. Winter hardening of Norwegian perennial forage crops in a prolonged growing season (Doctoral thesis). Norwegian University of Life Sciences, Ås.

de Boer, H.C., Deru, J.G.C., Hoekstra, N.J., van Eekeren, N., 2016. Strategic timing of nitrogen fertilization to increase root biomass and nitrogen-use efficiency of Lolium perenne L. Plant Soil 407, 81–90. https://doi.org/10.1007/s11104-016-2917-y

Du, L., Arauzo, P.J., Meza Zavala, M.F., Cao, Z., Olszewski, M.P., Kruse, A., 2020. Towards the Properties of Different Biomass-Derived Proteins via Various Extraction Methods. Molecules 25, 488. https://doi.org/10.3390/molecules25030488

Ergon, Å., Kirwan, L., Bleken, M.A., Skjelvåg, A.O., Collins, R.P., Rognli, O.A., 2016. Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. Grass Forage Sci. 71, 667–682. https://doi.org/10.1111/gfs.12250

Fowler, D., Coyle, M., Skiba, U., Sutton, M.A., Cape, J.N., Reis, S., Sheppard, L.J., Jenkins, A., Grizzetti, B., Galloway, J.N., Vitousek, P., Leach, A., Bouwman, A.F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., Voss, M., 2013. The global nitrogen cycle in the twenty-first century. Philos. Trans. R. Soc. B Biol. Sci. 368, 20130164. https://doi.org/10.1098/rstb.2013.0164

Freibauer, A., Kaltschmitt, M., 2003. Controls and models for estimating direct nitrous oxide emissions from temperate and sub-boreal agricultural mineral soils in Europe. Biogeochemistry 63, 93–115. https://doi.org/10.1023/A:1023398108860

Fuchs, K., Merbold, L., Buchmann, N., Bellocchi, G., Bindi, M., Brilli, L., Conant, R.T., Dorich, C.D., Ehrhardt, F., Fitton, N., Grace, P., Klumpp, K., Liebig, M., Lieffering, M., Martin, R., McAuliffe, R., Newton, P.C.D., Rees, R.M., Recous, S., Smith, P., Soussana, J., Topp, C.F.E., Snow, V., 2020. Evaluating the potential of legumes to mitigate N_2O emissions from permanent grassland using process-based models. Glob. Biogeochem. Cycles 34, 1–36. https://doi.org/10.1029/2020GB006561

Gaudin, A., Westra, S., Loucks, C., Janovicek, K., Martin, R., Deen, W., 2013. Improving Resilience of Northern Field Crop Systems Using Inter-Seeded Red Clover: A Review. Agronomy 3, 148–180. https://doi.org/10.3390/agronomy3010148

Guerrero, J.-L., Sample, J.E., 2022. Kildefordelte tilførsler av nitrogen og fosfor til norske kystområder i 2020 – tabeller, figurer og kart (No. 7729–2022, in Norwegian). Norsk institutt for vannforskning.
Hansen, S., Bernard, M.-E., Rochette, P., Whalen, J.K., Dörsch, P., 2014. Nitrous oxide emissions from a fertile grassland in Western Norway following the application of inorganic and organic fertilizers. Nutr. Cycl. Agroecosystems 98, 71–85. https://doi.org/10.1007/s10705-014-9597-x
Hansen, E.M., Eriksen, J., 2016. Nitrate leaching in maize after cultivation of differently managed grass-clover leys on coarse sand in Denmark. Agriculture, Ecosystems & Environment 216, 309–313. https://doi.org/10.1016/j.agee.2015.10.010
Hernandez, P., Picon-Cochard, C., 2016. Presence of Trifolium repens Promotes Complementarity of Water Use and N Facilitation in Diverse Grass Mixtures. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00538
Hoekstra, N.J., Finn, J.A., Buchmann, N., Gockele, A., Landert, L., Prill, N., Scherer-Lorenzen, M., Lüscher, A., 2014a. Methodological tests of the use of trace elements as tracers to assess root activity. Plant Soil 380, 265–283. https://doi.org/10.1007/s11104-014-2048-2
Hoekstra, N.J., Finn, J.A., Hofer, D., Lüscher, A., 2014b. The effect of drought and interspecific interactions on depth of water uptake in deep- and shallow-rooting grassland species as determined by $\delta\,^{18}O$ natural abundance. Biogeosciences 11, 4493–4506. https://doi.org/10.5194/bg-11-4493-2014
Hoekstra, N.J., Suter, M., Finn, J.A., Husse, S., Lüscher, A., 2015. Do belowground vertical niche differences between deep- and shallow-rooted species enhance resource uptake and drought resistance in grassland mixtures? Plant Soil 394, 21–34. https://doi.org/10.1007/s11104-014-2352-x
Höglind, M., Cameron, D., Persson, T., Huang, X., van Oijen, M., 2020. BASGRA_N: A model for grassland productivity, quality and greenhouse gas balance. Ecol. Model. 417, 108925. https://doi.org/10.1016/j.ecolmodel.2019.108925
Hooper, D.U., 1998. The role of complementarity and competition in ecosystem responses to variation in plant diversity. Ecology 79, 704–719. https://doi.org/10.1890/0012-9658(1998)079[0704:TROCAC]2.0.CO;2
Husse, S., Lüscher, A., Buchmann, N., Hoekstra, N.J., Huguenin-Elie, O., 2017. Effects of mixing forage species contrasting in vertical and temporal nutrient capture on nutrient yields and fertilizer recovery in productive grasslands. Plant Soil 420, 505–521. https://doi.org/10.1007/s11104-017-3372-0
IPCC, 2022. Summary for Policymakers. In: Climate Change 2022: Mitigation of Climate Change. Contribution of Working Group III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [P.R. Shukla, J. Skea, R. Slade, A. Al Khourdajie, R. van Diemen, D. McCollum, M. Pathak, S. Some, P. Vyas, R. Fradera, M. Belkacemi, A. Hasija, G. Lisboa, S. Luz, J. Malley, (eds.)]. Cambridge University Press, Cambridge, UK and New York, NY, USA. doi: 10.1017/9781009157926.001.
Jumpponen, A., Högberg, P., Huss-Danell, K., Mulder, C.P.H., 2002. Interspecific and spatial differences in nitrogen uptake in monocultures and two-species mixtures in north European grasslands: *Nitrogen uptake in north European grasslands*. Funct. Ecol. 16, 454–461. https://doi.org/10.1046/j.1365-2435.2002.00642.x

Klima- og miljødepartementet, 2021. Norway's Climate Action Plan for 2021–2030 — Meld. St. 13 (2020–2021) Report to the Storting (white paper).
Koga, S., Böcker, U., Uhlen, A.K., Hoel, B., Moldestad, A., 2016. Investigating environmental factors that cause extreme gluten quality deficiency in winter wheat (*Triticum aestivum* L.). Acta Agric. Scand. Sect. B — Soil Plant Sci. 66, 237–246. https://doi.org/10.1080/09064710.2015.1088570
Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World Map of the Köppen-Geiger climate classification updated. Meteorol. Z. 15, 259–263. https://doi.org/10.1127/0941-2948/2006/0130
Kristensen, H.L., Thorup-Kristensen, K., 2007. Effects of vertical distribution of soil inorganic nitrogen on root growth and subsequent nitrogen uptake by field vegetable crops. Soil Use Manag. 23, 338–347. https://doi.org/10.1111/j.1475-2743.2007.00105.x
Kunhikrishnan, A., Thangarajan, R., Bolan, N.S., Xu, Y., Mandal, S., Gleeson, D.B., Seshadri, B., Zaman, M., Barton, L., Tang, C., Luo, J., Dalal, R., Ding, W., Kirkham, M.B., Naidu, R., 2016. Functional Relationships of Soil Acidification, Liming, and Greenhouse Gas Flux, in: Advances in Agronomy. Elsevier, pp. 1–71. https://doi.org/10.1016/bs.agron.2016.05.001
Landbruksdirektoratet, 2021. Bruk av norske fôrressurser. Utredning av forbedring av virkemidler med sikte på økt produksjon og bruk av norsk fôr. (No. 10/2021, in Norwegian).
Landrø Hjelt, A., Jenssen, E., Hansen, Ø., Ystad, E., Olsen, A., 2019. Økonomien i grasbasert melk- og kjøttproduksjon i Nord-Norge (No. 5/140/2019). Norsk institutt for bioøkonomi.
Leep, R.H., 1989. Improving pastures in Michigan by frost seeding. Extension Bulletin E-2185. Michigan State University, Cooperative Extension Service.
Li, M., Shimizu, M., Hatano, R., 2015. Evaluation of N_2O and CO_2 hot moments in managed grassland and cornfield, southern Hokkaido, Japan. CATENA 133, 1–13. https://doi.org/10.1016/j.catena.2015.04.014
Liu, B., Frostegård, Å., Bakken, L.R., 2014. Impaired Reduction of N_2O to N_2 in Acid Soils Is Due to a Posttranscriptional Interference with the Expression of nosZ. mBio 5, e01383-14-e01383-14. https://doi.org/10.1128/mBio.01383-14
Malcolm, B.J., Moir, J.L., Cameron, K.C., Di, H.J., Edwards, G.R., 2015. Influence of plant growth and root architecture of Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) on N recovery during winter. Grass Forage Sci. 70, 600–610. https://doi.org/10.1111/gfs.12157
Mamolos, A.P., Elisseou, G.K., Veresoglou, D.S., 1995. Depth of Root Activity of Coexisting Grassland Species in Relation to N and P Additions, Measured Using Nonradioactive Tracers. J. Ecol. 83, 643. https://doi.org/10.2307/2261632
McCool, D.K., Molnau, M., 1984. Measurement of Frost Depth, in: Proceedings Western Snow Conference 52. pp. 33–42.
Miljødirektoratet, 2022. The Norwegian Pollutant Release and Transfer Register. Norwegian Environment Agency, Trondheim, Norway. Accessed 22 December, 2022.
Milovanović, J., Eich-Greatorex, S., Krogstad, T., Rakic, V., Rajic, N., 2015. The use in grass production of clinoptilolite as an ammonia adsorbent and a nitrogen

carrier. J. Serbian Chem. Soc. 80, 1203–1214. https://doi.org/10.2298/JSC150317042M

Mommer, L., Van Ruijven, J., De Caluwe, H., Smit-Tiekstra, A.E., Wagemaker, C.A.M., Joop Ouborg, N., Bögemann, G.M., Van Der Weerden, G.M., Berendse, F., De Kroon, H., 2010. Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species: Below-ground species distributions in a biodiversity experiment. J. Ecol. 98, 1117–1127. https://doi.org/10.1111/j.1365-2745.2010.01702.x

Mørkved, P.T., Dörsch, P., Henriksen, T.M., Bakken, L.R., 2006. N_2O emissions and product ratios of nitrification and denitrification as affected by freezing and thawing. Soil Biol. Biochem. 38, 3411–3420. https://doi.org/10.1016/j.soilbio.2006.05.015

Nadeem, S., Bakken, L.R., Frostegård, Å., Gaby, J.C., Dörsch, P., 2020. Contingent Effects of Liming on N_2O-Emissions Driven by Autotrophic Nitrification. Front. Environ. Sci. 8, 598513. https://doi.org/10.3389/fenvs.2020.598513

Nørgaard, DS, Stærmose, MD & Jensen, PR 2018, Protein from green biomass as a food resource. in CM & KM (eds), Sustain Conference 2018: Creating Technology for a Sustainable Society., P-1, Technical University of Denmark, Lyngby, Denmark, Sustain conference 2018: Creating Technology for a Sustainable Society, Kongens Lyngby, Denmark, 29/11/2018. <http://www.sustain.dtu.dk/>

Nyfeler, D., Huguenin-Elie, O., Suter, M., Frossard, E., Lüscher, A., 2011. Grass–legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. Agric. Ecosyst. Environ. 140, 155–163. https://doi.org/10.1016/j.agee.2010.11.022

Østrem, L., Helgadottir, A., Niemeläinen, O., Seppänen, M., Greve Pedersen, M., Halling, M., Rognli, O.A., 2015a. Environmental impact on winter survival and production in non-native grasses in the Nordic countries. Presented at the Proceedings of the 25th NJF Congress, Nordic View to Sustainable Rural Development, pp. 127–133.

Østrem, L., Rapacz, M., Larsen, A., Dalmannsdottir, S., Jørgensen, M., 2015b. Influences of growth cessation and photoacclimation on winter survival of non-native Lolium–Festuca grasses in high-latitude regions. Environ. Exp. Bot. 111, 21–31. https://doi.org/10.1016/j.envexpbot.2014.10.008

Parton, W.J., Holland, E.A., Del Grosso, S.J., Hartman, M.D., Martin, R.E., Mosier, A.R., Ojima, D.S., Schimel, D.S., 2001. Generalized model for NO_x and N_2O emissions from soils. J. Geophys. Res. Atmospheres 106, 17403–17419. https://doi.org/10.1029/2001JD900101

Pirhofer-Walzl, K., Eriksen, J., Rasmussen, J., Høgh-Jensen, H., Søegaard, K., Rasmussen, J., 2013. Effect of four plant species on soil ^{15}N-access and herbage yield in temporary agricultural grasslands. Plant Soil 371, 313–325. https://doi.org/10.1007/s11104-013-1694-0

Powlson, D.S., Barraclough, D., 1993. Mineralization and Assimilation in Soil–Plant Systems, in: Nitrogen Isotope Techniques. Elsevier, pp. 209–242. https://doi.org/10.1016/B978-0-08-092407-6.50013-4

Prosser, J.I., Hink, L., Gubry-Rangin, C., Nicol, G.W., 2020. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. Glob. Change Biol. 26, 103–118. https://doi.org/10.1111/gcb.14877

Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous Oxide (N_2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. Science 326, 123–125. https://doi.org/10.1126/science.1176985

Rees, R.M., Augustin, J., Alberti, G., Ball, B.C., Boeckx, P., Cantarel, A., Castaldi, S., Chirinda, N., Chojnicki, B., Giebels, M., Gordon, H., Grosz, B., Horvath, L., Juszczak, R., Kasimir Klemedtsson, å., Klemedtsson, L., Medinets, S., Machon, A., Mapanda, F., Nyamangara, J., Olesen, J.E., Reay, D.S., Sanchez, L., Sanz Cobena, A., Smith, K.A., Sowerby, A., Sommer, M., Soussana, J.F., Stenberg, M., Topp, C.F.E., van Cleemput, O., Vallejo, A., Watson, C.A., Wuta, M., 2013. Nitrous oxide emissions from European agriculture – an analysis of variability and drivers of emissions from field experiments. Biogeosciences 10, 2671–2682. https://doi.org/10.5194/bg-10-2671-2013

Reinsch, T., Loges, R., Kluß, C., Taube, F., 2018. Renovation and conversion of permanent grass-clover swards to pasture or crops: Effects on annual N_2O emissions in the year after ploughing. Soil Tillage Res. 175, 119–129. https://doi.org/10.1016/j.still.2017.08.009

Risk, N., Snider, D., Wagner-Riddle, C., 2013. Mechanisms leading to enhanced soil nitrous oxide fluxes induced by freeze–thaw cycles. Can. J. Soil Sci. 93, 401–414. https://doi.org/10.4141/cjss2012-071

Sandve, S.R., Kosmala, A., Rudi, H., Fjellheim, S., Rapacz, M., Yamada, T., Rognli, O.A., 2011. Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. Plant Sci. 180, 69–77. https://doi.org/10.1016/j.plantsci.2010.07.011

Schmid, B., Hector, A., Saha, P., Loreau, M., 2008. Biodiversity effects and transgressive overyielding. J. Plant Ecol. 1, 95–102. https://doi.org/10.1093/jpe/rtn011

Smith, K.A., Dobbie, K.E., Thorman, R., Watson, C.J., Chadwick, D.R., Yamulki, S., Ball, B.C., 2012. The effect of N fertilizer forms on nitrous oxide emissions from UK arable land and grassland. Nutr. Cycl. Agroecosystems 93, 127–149. https://doi.org/10.1007/s10705-012-9505-1

SSB, 2022. Jordbruksareal (dekar), etter vekst, statistikkvariabel og år (No. 05982, in Norwegian). Statistisk sentralbyrå, Norway.

Sturite, I., Henriksen, T.M., Breland, T.A., 2007. Longevity of White Clover (Trifolium repens) Leaves, Stolons and Roots, and Consequences for Nitrogen Dynamics under Northern Temperate Climatic Conditions. Ann. Bot. 100, 33–40. https://doi.org/10.1093/aob/mcm078

Sturite, I., Rivedal, S., Dörsch, P., 2021. Clover increases N_2O emissions in boreal leys during winter. Soil Biol. Biochem. 163, 108459. https://doi.org/10.1016/j.soilbio.2021.108459

Sturite, I., Uleberg, M.V., Henriksen, T.M., Jørgensen, M., Bakken, A.K., Breland, T.A., 2006. Accumulation and Loss of Nitrogen in White Clover (Trifolium repens L.) Plant Organs as Affected by Defoliation Regime on Two Sites in Norway. Plant Soil 282, 165–182. https://doi.org/10.1007/s11104-005-5697-3

Thuen, A.E., Tufte, T., 2019. Grasbasert ammekuproduksjon - Tiltak for økt bruk av grovfôr (No. 7–2019, in Norwegian). AgriAnalyse, Oslo, Norway.
Tian, H., Yang, J., Xu, R., Lu, C., Canadell, J.G., Davidson, E.A., Jackson, R.B., Arneth, A., Chang, J., Ciais, P., Gerber, S., Ito, A., Joos, F., Lienert, S., Messina, P., Olin, S., Pan, S., Peng, C., Saikawa, E., Thompson, R.L., Vuichard, N., Winiwarter, W., Zaehle, S., Zhang, B., 2019. Global soil nitrous oxide emissions since the preindustrial era estimated by an ensemble of terrestrial biosphere models: Magnitude, attribution, and uncertainty. Glob. Change Biol. 25, 640–659. https://doi.org/10.1111/gcb.14514
Vazquez De Aldana, B.R., Berendse, F., 1997. Nitrogen-use efficiency in six perennial grasses from contrasting habitats. Funct. Ecol. 11, 619–626. https://doi.org/10.1046/j.1365-2435.1997.00137.x
Volden, H., 2019. Faglige utfordringer når mjølkevolumet reduseres med 100 millioner liter [WWW Document]. URL https://medlem.tine.no/fag-og-forskning/faglige-utfordringer-n%C3%A5r-mj%C3%B8lkevolumet-reduseres-med-100-millioner-liter (accessed 12.23.22).
Wagner-Riddle, C., Congreves, K.A., Abalos, D., Berg, A.A., Brown, S.E., Ambadan, J.T., Gao, X., Tenuta, M., 2017. Globally important nitrous oxide emissions from croplands induced by freeze–thaw cycles. Nat. Geosci. 10, 279–283. https://doi.org/10.1038/ngeo2907
Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2018. Meteorologiske data for Ås 2017. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Paper I

Deep N acquisition in hemiboreal cultivated grasslands: I. Species in pure stands

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina A. Bleken

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Abstract

Aims To develop a methodology to study the uptake and redistribution by plant activity of NH_4^+ from deep soil and to apply it to investigate deep root N uptake by cultivated grassland species.
Methods A slow-release $^{15}NH_4^+$ label adsorbed to clinoptilolite was placed in the soil (depth 42 cm) well below the densest root zone, in well-established pure stands of grass and clover species. The label was placed in early spring and its recovery tracked throughout the growing season in two repeated experiments.
Results After two growing seasons ~90% of the label was accounted for in the herbage and soil of the grass subplots, less in clover subplots. Uptake in harvested herbage was weak in spring and strong in summer and autumn. Transport of ^{15}N to the upper soil layers was largely due to plant activity. Species differed significantly in ability to recover and maintain ^{15}N in the soil-plant system. Ryegrass, timothy, meadow fescue and tall fescue herbage recovered ~65% of the ^{15}N label, bluegrass 54%, and white and red clover 36-48% in one growing season.
Conclusions The innovative slow-release ^{15}N label effectively enabled following plant uptake of NH_4^+ from deep soil. Deep N uptake was seasonal for all species, also tap-rooting red clover, in that ^{15}N recovery was limited in spring, increased mid-season, and was strong considering N deficiency and low yields in autumn. Tall fescue did not exhibit superior ability to recover deep NH_4^+ under severe drought. Ryegrass compensated for poor winter survival with vigorous summer ^{15}N uptake.

1. Introduction

There is an increasing demand for high quality forage grassland yields with high protein content and thus high N content, produced with high nitrogen use efficiency (NUE) and reduced losses. Higher yields and higher protein content are largely achieved by increasing N fertilization, which tends to reduce NUE and increase N dissipation to the environment. Mineral N fertilizer is often transported below the densest root zone, which is roughly 0-20 cm in a typical Norwegian forage grassland (Bleken et al., 2022). Perennial grassland species grown for forage differ in rooting depth (Craine et al., 2001) and in their ability to recover N from deeper soil (Hoekstra et al., 2015; Jumpponen et al., 2002; Pirhofer-Walzl et al., 2013).

Plant phenotypical response must be seen as a result of genome, environment and management (Großkinsky et al., 2015), with multiple cues and behaviors influencing root position (Cahill and McNickle, 2011; Hodge, 2004). Moreover, N uptake is generally seasonal and corresponds to growth vigor; for example, plants take up less N in early spring but rapidly increase uptake as growth conditions improve (Murphy et al., 2013). Many studies have shown that purportedly deep-rooting grassland species can access N from deep soil layers (Hoekstra et al., 2015; Kristensen and Thorup-Kristensen, 2004). Combining species with varied rooting depths is theorized to enhance nitrogen utilization throughout the soil profile due to vertical niche differentiation, although this is questioned (Hoekstra et al., 2015; Ravenek et al., 2014; Pirhofer-Walzl et al., 2013; von Felten et al., 2012; Mommer et al., 2010; von Felten and Schmid, 2008; Mamolos and Veresoglou, 2000). There are few studies on the relative ability of grassland species to take up nutrients from deeper soil, and it is even less clear how this behavior changes at different times throughout the growing season (Chen et al., 2016). We are not aware of field studies exploring how acquisition of deep N changes throughout the season.

Many rooting depth studies using ^{15}N applied it to the soil as $^{15}NO_3^-$, which is rapidly immobilized and lost by leaching (Kristensen and Thorup-Kristensen, 2004). Solutions of $^{15}NH_4^+$, which more readily adsorbs to soil particles though it is prone to immobilization and nitrification, may somewhat prolong label presence in the study system (Davis et al., 2006). In a unique approach, von Felten et al. (2012) used enriched ^{15}N plant litter as slow-release ^{15}N source in pots and harvested plants up to 11 months

later. Otherwise, we are not aware of previous experiments following the recovery of ^{15}N over several subsequent harvests after a single application. To further impede microbial immobilization, remineralization and leaching of the applied label we developed a novel labeling method using clinoptilolite, a natural zeolite which adsorbs and desorbs NH_4^+ via ion exchange with pseudo-second-order kinetics (Milovanović et al., 2015). This provided a slow-release label which allowed us to follow the fate of a single ^{15}N dose over more than a growing season, overcoming the common limitation that biomass must be harvested and analyzed within days or weeks of ^{15}N application.

The slow-release ^{15}N label enabled us to track uptake of NH_4^+ from a known depth over several successive harvests. We placed it in early spring in well-established grassland swards, at 42 cm depth in a naturally compact soil layer, markedly below the ploughing depth (20 cm) and the densest root zone, and followed the recovery of ^{15}N in subsequent harvests (3 cuts per year). The experiment was performed twice. In this article we present how deep N uptake in pure stands related to purported rooting depth and weather events. Niche and overyielding effects in mixtures were also explored in the same field experiment and the results are presented in a companion article (Byers et al., Manuscript II of this thesis).

Treatments included pure stands of seven species differing in expected rooting depths, N acquisition affinity, and persistence under Norwegian conditions. Timothy (*Phleum pratense* L. is the most common ley species in Nordic countries due to its winter hardiness and good forage quality. Meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.) is also commonly grown in Norway together with timothy and has better forage quality. It is expected have shallower roots than tall fescue. Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort) is considered to be a deep-rooting species (e.g. Hernandez and Picon-Cochard, 2016; Malcolm et al., 2015), drought resistant, and well adapted to Nordic conditions. Perennial ryegrass (*Lolium perenne* L.) is commonly described as fast establishing and shallow rooting (Finn et al., 2013); it is reputed for its high yields and nutritional value but overwinters poorly in Norway (Østrem et al., 2015). Kentucky bluegrass (*Poa pratensis* L.) is more common in pastures than forage mixtures and is known for lower yields but high N concentration and a shallow rhizomatous root system. Red and white clover (*Trifolium pratense* L., *Trifolium repens* L. cv.) were included for their N-fixing effect. Red clover is reputed to be deep-

rooting due to its persistent taproot structure (e.g. Ergon et al., 2016). White clover is by comparison shallow-rooting, forming stolons.

We also explored whether moderate N deficiency might alter N uptake from deeper soil. To do this we took into account that the minimum N concentration in the herbage at which plants can express potential growth (critical N concentration, N_{crit}) decreases with increasing plant size due to the larger proportion of fibers and thicker cell walls (e.g. Baadshaug and Lantinga, 2002; Bélanger and Gastal, 2000; Lemaire et al., 1984). This effect can lead to misinterpretation if N concentrations as such are taken to represent the N nutritional status when comparing stands with different herbage yields.

The following hypotheses were tested: (1) A single application of $^{15}NH_4^+$-enriched clinoptilolite can be used to effectively trace deep root N uptake over several harvests; (2) Generally, the recovery of deep-placed ^{15}N will correlate with growth vigor, and thus be stronger in the spring and summer than in the autumn; (3) Plant N deficiency will enhance the uptake of deep-placed ^{15}N; (4) Species reputed as deeper rooting will recover more ^{15}N from 42 cm depth, and nitrogen fixers less. Therefore, the annual recovery of deep-placed ^{15}N by pure stands from greatest to least will be: tall fescue, meadow fescue and timothy, perennial ryegrass, red clover, bluegrass, and white clover; (5) In ryegrass the annual recovery of deep-placed ^{15}N will strongly decline from year to year due to poor winter persistence in high latitudes.

2. Materials and methods

2.1. Site

The experiment was established in an Umbric Epistagnic Retisol (IUSS, 2015) at the NMBU research farm in Ås, Norway (59°39'49"N, 10°45'38"E, 69 m a.s.l.). The soil is a stone-free silty loam (~31% clay, 44% silt and 25% sand), artificially drained at 1 m depth. The bulk density is low in the top layer (1.15 g cm^{-2}), but below the ploughing depth (20 cm) it increases markedly to 1.5 g cm^{-2} at 40 cm (Suppl. Table S 1). During the growing season from May through September, the mean temperature normal (1991-2020) is 13.8°C, while it was 14.6°C and 13.7°C in 2016 and 2017, respectively. For

October through April, the mean temperature normal is 0.9°C, while it was 1.6°C and 1.5°C during the winters leading to spring 2016 and 2017. During the winter, freeze-thaw events and periods with snow cover are common. The annual precipitation normal is 892 mm, roughly evenly distributed through the year, but periods of drought can occur, especially in early summer due to strong insolation and wind. Weather data are from the nearby weather station Ås (No. SN17850) at 59°39'37.8" N, 10°46'54.5" E, 94 m a.s.l. (Wolff et al., 2021, 2018, 2017, 2016).

2.2. Crop management and experimental treatments

Pure stands of perennial ryegrass (*Lolium perenne* L. cv. Figgjo), tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort. cv. Swaj), meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv. cv. Fure), timothy (*Phleum pratense* L. cv. Grindstad), Kentucky bluegrass red clover (*Trifolium pratense* L. cv. Lea) and white clover (*Trifolium repens* L. cv. Milkanova) were established in June 2014, on 12 sowing rows/plot spaced 12 cm apart. The best locally-adapted cultivars were chosen. The treatment plots (8 m x 1.5 m) were replicated 4 or more times and fully randomized among several treatments, including grass-clover mixtures which will be considered in a companion article (Byers et al., Manuscript II of this Thesis).

In the following years the swards were harvested three times per year: in spring (H1) when 10% of timothy inflorescence started to be visible, then in summer (H2) after 600-650 growing degree days (basis temperature 0°C), and in autumn (H3) around mid-September (Table 1) following local recommendations for high forage digestibility. All treatments received a moderate N fertilizer dose, in total 200 kg N ha^{-1} yr^{-1} applied as NPK 22-3-10, distributed 40% in early spring before H1, 40% after H1 and 20% after H2 (Table 1). Red and white clover seed was inoculated using soil from an organically managed crop rotation.

Table 1: Dates of ^{15}N label applications L1 and L2, herbage harvests and N fertilization. For each harvest, cumulated rainfall + irrigation in mm and growing degree days, basis 5°C from 1 April, are given for the period spanning from the previous harvest or 1 April for H1.

Operation	2016 (2nd prod. year)	2017 (3rd prod. year) [a]	2018 (4th prod. year) [a]
80 kg N ha^{-1}	25 April	2 May	27 April [c]
^{15}N application	L1: 26-28 April	L2: 18-20 April	
H1: Spring harvest	1 June (141 mm, 480 GDD)	6 June (125 mm, 499 GDD)	30 May (65+30 mm, 556 GDD)
80 kg N ha^{-1}	3 June	19 June	
H2: Summer harvest	12 July (118+15 mm, 637 GDD)	19 July [b] (107+15 mm, 640 GDD)	
40 kg N ha^{-1}	21 July	27 July	
H3: Autumn harvest	2 Sept. (166 mm, 821 GDD)	18 Sept. (260 mm, 884 GDD)	

[a] L1 subplots were harvested three additional times in 2017, and L2 subplots were harvested one additional time in 2018; these results are available in Suppl. Table S 5.
[b] During a drought period from 11 June to 10 July 2017 there was only 32 mm of precipitation and no irrigation until just before H2.
[c] April fertilization in 2018 was 120 kg N ha^{-1}.

2.3. ^{15}N Labeling

Two ^{15}N labeling experiments were established before the onset of regrowth in the early spring of the second (L1: 26-28 April 2016) and third production years (L2: 18-20 April 2017). Four labeled subplots were distributed each spring in four replicates of each treatment. In L2 some subplots were placed on plots used also in L1, in which case they were at least 5 m apart. In L2 a de-vegetated treatment was added wherein subplots were kept clear of vegetation for the whole season.

The label was prepared by shaking clinoptilolite for 24 hours in a 98 atom percent (AT%) $^{15}NH_4Cl$ solution, before drying and extracting it for NH_4-N (0.5 g clinoptilolite in 25 mL 2*M* KCl) to determine the amount adsorbed.

Each subplot received a dose of 36 mg ^{15}N, which translates to 68 mg m^{-2} of plant sampling area (See 2.4. ^{15N} recovery by plants and redistribution in the soil). To label

subplots, we pre-augured a 4 x 4 array of 16 mm diameter, 0.43 m deep holes centered between sowing rows and spaced 12 cm apart (Fig. 1). The ^{15}N-labeled clinoptilolite was introduced through a PVC pipe to fill ~41-43 cm depth (1 g hole^{-1} in L1 and 0,87 g hole^{-1} in L2). Dry, finely sieved soil from the same field was used to flush the pipe before removing, and after its removal the hole was filled with more dry soil which was carefully compacted. This was done to prevent preferential root growth, as this clayey soil expands on rewetting.

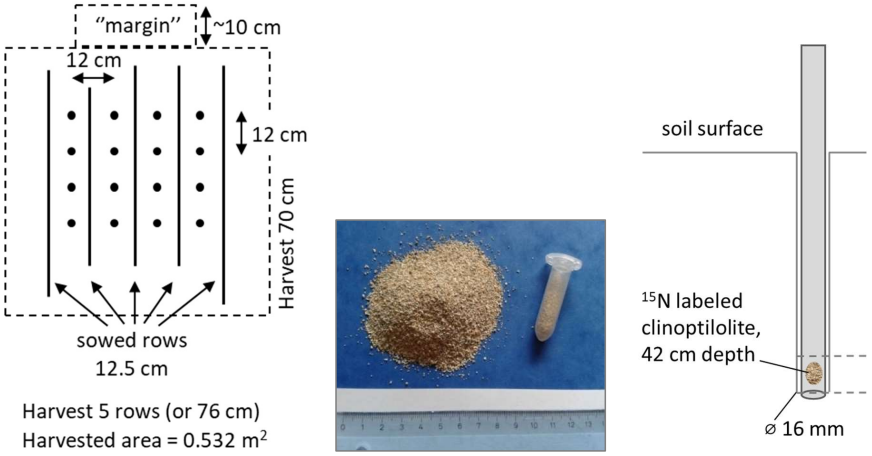


Figure 1: From left: Placement and harvest area of ^{15}N labeled subplots; Photograph of ^{15}N-labeled clinoptilolite and vial containing one of 16 doses per plot; Depth and method of placement of clinoptilolite

2.4. ^{15}N recovery by plants and redistribution in the soil

Herbage above ~5 cm stubble height was collected at each harvest (See 2.2. Crop management and experimental treatments) from five sow rows (or 76 cm in non-row forming white clover and bluegrass) along 70 cm (Fig. 1). The plant sampling area, 0.532 m^2, was larger than the 48 x 48 cm area immediately surrounding the ^{15}N label placement; this was done to assure recovery of as much label as possible (Powlson and Barraclough, 1993). To check that the harvest area was adequate, in L1 we also sampled herbage from an adjacent 10 cm wide margin, which was assumed to be representative for the whole perimeter (0.3 m^2). All herbage was dried at 40-60°C under ventilation, chopped and a subsample was ground and ball-milled.

Passive upward ^{15}N redistribution by diffusion and convection in the soil profile was investigated in the de-vegetated subplots in L2. At labeling the projection of the clinoptilolite position on the soil surface was marked, and soil was sampled one and five months later, with the auger placed in between the positions of the clinoptilolite. All soil samples for ^{15}N analysis were sieved to 2.0 mm and oven-dried at 60°C under strong ventilation, and a representative subsample was pulverized in an agate mill.

Approximately 17 months after labeling in L1, redistribution of ^{15}N in the soil profile in the presence of plants was studied to 60 cm depth on 9-12 October 2017 (Suppl. Table S 1). The thickness of the layers was adjusted to center around the clinoptilolite, after taking into consideration the ploughing depth (20 cm), below which soil organic matter (SOM) decreases abruptly, and the soil becomes very compact (Suppl. Table S 1). Four to five soil cores per subplot were taken on the planting rows to avoid sampling directly on the clinoptilolite and pooled together.

2.5. Ancillary observations: soil mineral N content and bulk density

Soil mineral N content (N_{min}) was analyzed in the soil removed when preparing the holes before label placement (only at 30-43 cm depth in L1, and 0-20, 20-30 and 30-43 cm depths in L2) and again in autumn (26 October 2016 and 30 October 2017). In the autumn soil samples were taken from non-labeled areas of the treatment plots using a hydraulic press (0-20, 20-40 and 40-60 cm depths, four soil cores per plot). The soil samples were immediately refrigerated to 4°C, and either the same day or within two days sieved to 2.0 mm. Sieved samples were either immediately extracted for KCl-extractable NH_4, NO_2 and NO_3-N, or immediately frozen and extracted later, adding the extractant before thawing. Soil extracts were frozen and analyzed within one month by colorimetric assay on a Tecan Infinite F50 microplate photometer (See Suppl. Table S 2, Byers et al., 2021).

The soil bulk density (BD) was measured using 100 cm^3 intact cores taken on 1 November 2017 at corresponding depths from four locations within the field (Suppl. Table S 1).

2.6. ^{15}N analysis and recovery calculations

Nitrogen content and ^{15}N abundance were analyzed using a Thermo 1112 HT flash combustion element analyzer, coupled through a Finnigan ConFlo III to a Finnigan Delta Plus XP isotope ratio mass spectrometer (IRMS). Atom percent (AT%) of ^{15}N was corrected for scale and drift using house standards which had been calibrated to the international standards IAEA-N1 and N2, and USGS32 and 34. The N content was quantified by calibrating peak area of m/z 28 against known amounts of EDTA.

To calculate excess ^{15}N AT% above natural abundance, we subtracted the AT% of unlabeled samples taken from the same field (timothy, 0.3690 AT%, for the grasses; white clover, 0.3686 AT%, for the clovers; natural abundance by depth for soil samples are given in Suppl. Table S 1).

The label ^{15}N recovered in herbage and soil samples was calculated as:

$$Excess\ mg\ ^{15}N\ g^{-1}N = \frac{15(Excess\ AT\%\ ^{15}N)}{15(AT\%\ ^{15}N) + 14(1 - AT\%\ ^{15}N)}\ (10^3) \quad (1)$$

^{15}N uptake by aboveground herbage (DM) per area was calculated as:

$$Herbage\ uptake\ mg\ ^{15}N\ m^{-2} = (Excess\ mg\ ^{15}N\ g^{-1}N)\ (g\ N\ g^{-1}DM)\ (g\ DM\ m^{-2}) \quad (2)$$

Since the ^{15}N uptake in the herbage at H2 and H3 depended on the amount left in the soil after the previous harvests, the ^{15}N recovery strength (Eqn. 3) was weighted plotwise, assuming plant uptake into the harvested herbage to be the only removal of label ^{15}N:

$$^{15}N\ recovery\ strength = \left(\frac{Herbage\ uptake\ g\ ^{15}N_{\ current\ harvest}}{Applied\ g\ ^{15}N - \Sigma(Herbage\ uptake\ g\ ^{15}N)_{previous\ harvests}}\right) \quad (3)$$

As an indication for the plants' ability to utilize N_{min} from deeper soil to contribute to their total N acquisition, we estimated the Relative Deep Uptake Index (RDUI) as ^{15}N recovery strength per N yield:

$$RDUI = \left(\frac{^{15}N\ recovery\ strength_{current\ harvest}}{N\ yield_{current\ harvest}}\right) \quad (4)$$

We also estimated Recovery per Dry Matter (RDM) as ^{15}N recovery strength per DM yield:

$$RDM = \left(\frac{^{15}N\ recovery\ strength_{current\ harvest}}{DM\ yield_{current\ harvest}}\right) \quad (5)$$

Regarding the soil samples, the amount of ^{15}N label present in each soil layer was calculated:

$$mg\ ^{15}N\ layer^{-1} = \left(\frac{Excess\ mg\ ^{15}N}{g\ N}\right) * \left(\frac{g\ N}{g\ dry\ soil}\right) * (BD\ g\ cm^{-3}) * (Layer\ thickness\ cm) * (48x48\ cm^2) \quad (6)$$

The area was assumed to be 48 x 48 cm evenly spaced around the clinoptilolite placement (thus smaller than the herbage harvest area shown in Fig. 1). The measured bulk density (BD) was interpolated to match the exact depths of soil samples taken (2.5. Ancillary observations: soil mineral N content and bulk density).

2.7. Plant nutritional N status

To evaluate the plants' nutritional N status at the time of harvest, the critical N concentration (N_{crit}) for potential growth was estimated plotwise and for each harvest as a negative power function of the DM yield, adapted to Norwegian conditions (Baadshaug and Lantinga, 2002, Suppl. Text S 1). The plants' N status was then calculated as a ratio of the N concentration measured in the herbage (g N g^{-1} DM) and the N_{crit} estimated for each subplot, indicating N deficiency when < 1.

2.8. Statistics

For all response variables considered, analyses of variance were performed using the R software package, version 3.6.1. We used *post hoc* Tukey tests for multiple comparisons of species performance within a single harvest and cumulatively (using simple.glht from the mixlm package for R, α=0.05). Correlation analyses were performed using Proc Corr in SAS 9.4 (Statistical Analysis System; SAS Institute, NC, USA). Statistically significant differences ($p \leq 0.05$) are, for brevity, referred to as significant.

3. Results

3.1. Mineral N in the soil profile in the spring and autumn

In both years, the spring and autumn soil N_{min} content in the grass plots was only half or less the content in the clover plots, and there were no significant differences within the grasses or the clovers (Suppl. Table S 3 A). In the 30-43 cm soil layer, the initial spring N_{min} content in L1 was ~0.9 g m^{-2} in the grass and 2.5 g m^{-2} in the clover treatments, and half as much in L2. Thus, related to the plant sampling area, ^{15}N addition (See 2.3. ^{15N} Labeling) corresponded at most to ~15% of the N_{min} in the 40-43 cm layer, 2-5% of the total N_{min} in the spring (0-43 cm profile, measured only in L2) and ~0.8% of fertilizer N applied in the spring (See 2.2. Crop management and experimental treatments).

Grasses effectively depleted N_{min}, leaving $\lesssim$1 g N_{min} m^{-2} in the 0-60 cm soil profile by the end of each growing season. Although clovers were less effective, they left only $\lesssim$3 g N_{min} m^{-2} (Suppl. Table S 3 B).

3.2. Physical diffusion of ^{15}N and redistribution in the soil profile by plant activity

Our approach to follow the herbage uptake of one dose of deep-placed ^{15}N over three harvests assumes minimal upward diffusion of ^{15}N in the absence of plant roots and minimal leaching loss during the growing season. We tested this assumption in the de-vegetated subplots in L2. One month after labeling, at most 3% of the applied ^{15}N was found in the soil layer (38-46 cm depth) surrounding the ^{15}N-loaded clinoptilolite (42 cm depth), and even less in the adjacent layers below and above the label (Fig. 2 A). No

significant ^{15}N enrichment was found above 30 cm depth. After 5 months, 7% of the original label was found in the layer immediately below the clinoptilolite position, ~4% in the 38-46 cm layer centered around the clinoptilolite, ~2% in the layer immediately above it and less than 1% within the ploughing depth (0 to 24 cm, see Suppl. Table S 4 A). We conclude that despite higher water contents in the de-vegetated than the vegetated subplots (data not shown), leaching and diffusive redistribution upwards of ^{15}N were negligible.

Redistribution of the ^{15}N throughout the soil profile due to plant activity was explored in L1 after two growing seasons, i.e. 17 months or six harvests after labeling. On average ~22% of the applied ^{15}N was found in the soil profile, without significant differences between species (Fig. 2 B and C, Suppl. Table S 4 B). A strong vertical redistribution by plants was evident, as most of the remaining ^{15}N was found in the upper 10 cm of the soil (on average 43%), and nearly 60% in the upper 0-24 cm, while only ~12% (1-4% of the ^{15}N applied) was transported below the labeling depth to the 46-60 cm layer. Tall fescue tended to have the least residual soil ^{15}N below 20 cm depth.

Cumulated over the three successive harvests of L1, only 1.1 to 2% of the original ^{15}N label was recovered in herbage harvested from the 10-cm wide perimeter surrounding the sampling area, the most around tall fescue; no ^{15}N enrichment was detected in the margins of red clover plots (Suppl. Fig. S 1). This confirmed that the sampled area was sufficiently large to comprise most of the ^{15}N label recovered in aboveground herbage.

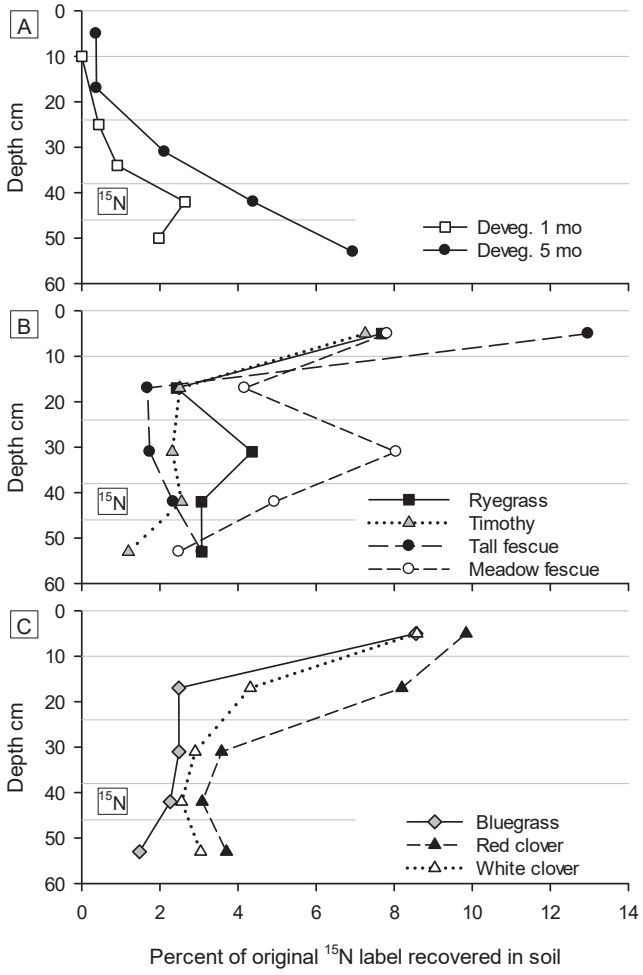


Figure 2: Percent of ^{15}N found in each soil layer, relative to the original amount of ^{15}N label placed at 42 cm depth. Mean values of 4 replicates; standard errors and p-values from one-sided t-tests are available in Suppl. Table S 4. Horizontal lines separate the soil layers sampled, with the ^{15}N recovery shown in the center of the layer.
A) **De-vegetatated subplots**, sampled 1 and 5 months after ^{15}N labeling (L2). A slightly different depth profile was sampled at 1 month because less movement of ^{15}N was expected. ^{15}N enrichment at 0-20 and 20-30 cm depths at 1 month were not significant, and ^{15}N enrichment at combined 0-24 cm depth at 5 months was <1% of the original label.
B and C) **Vegetated subplots**, sampled 17 months after ^{15}N labeling (L1). One ryegrass replicate at 38-46 cm was probably contaminated with ^{15}N-labeled clinoptilolite and was removed. The high mean ^{15}N content at 0-10 cm depth in tall fescue was due to one replicate, values were otherwise similar to those in other treatments.

3.3. Total ^{15}N accounted for in harvested herbage and soil

After two growing seasons, 17 months after labeling in L1, the sum of ^{15}N in herbage from six consecutive harvests plus ^{15}N present in the soil profile at the end of the period accounted for 85-90% of the ^{15}N applied in the subplots of perennial ryegrass, timothy and both fescues (four species hereafter called tall-growing grasses). Significantly less was detected in subplots of bluegrass (~70%), red clover (~75%), and white clover (~55%), mainly due to less ^{15}N in harvested herbage rather than differences in soil ^{15}N (Fig. 3). The ^{15}N in herbage from the fourth through sixth harvests of L1 subplots (the second year after labeling) was small: <5% of the original ^{15}N label in ryegrass, <10% in both fescues, up to 10-17% in the remaining species, and was found primarily in the fourth (spring) harvest. These harvests are not included in our evaluation of deep N utilization by different species but are available in Suppl. Table S 5.

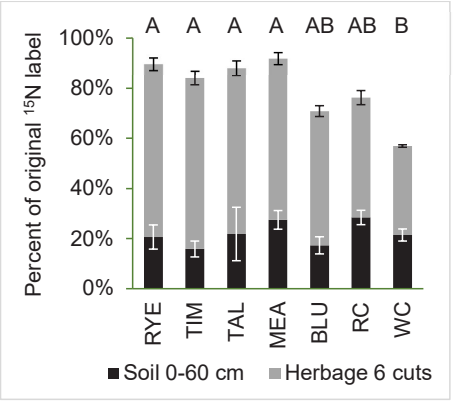


Figure 3: Percent of ^{15}N label recovered in herbage from two growing seasons and soil samples taken 17 months after L1. Mean of 4 replicates (±SE). Letters show *post hoc* Tukey groupings (α=0.10) of the summed ^{15}N in soil plus herbage. One replicate of ryegrass at 38-46 cm with an extremely high value, indicating the sample directly contained ^{15}N-labeled clinoptilolite, was omitted.

3.4: Growth conditions and plant performance

Both winters and early springs leading up to the growing seasons of L1 and L2 were harsh with repeated freeze-thaw events starting from mid-November (Suppl. Fig. S 2). Snow protected the leys in January before L1, when the mean air temperature was below −15°C for about a week, however there were intermittent thawing and cold spells with snow from mid-February to the end of March. Then in early spring, strong insolation and wind dried the soil, causing most severe drought in plots with little herbage mulch. The winter leading up to L2 was characterized by a warm December

(mean temperature 0.7°C), which reduces plant hardening, and no snow cover until February.

White clover barely survived the early pre-regrowth drought in L1, and therefore could not be harvested at H1 of L1; however, stolons survived and provided good regrowth for the subsequent harvests (Fig. 4 A). Spring regrowth of white clover was also poor in L2, though it could be harvested. Ryegrass was the grass species with poorest winter survival and most N-limitation in spring, both years and especially in L2 (Fig. 4 A, D). Timothy and bluegrass best survived the second winter. Timothy overwintered to L2 very well and had 10% higher yields in H1 of L2 than in H1 of L1 (Fig. 4 A). Both fescues overwintered well yet had reduced spring yields in L2 compared to L1 (Fig. 4 A), tall fescue more severely.

Soil moisture was overall adequate during the growing season of L1, including 15 mm irrigation on 13 June 2016 (Table 1). By contrast, the summer period of L2 was characterized by prolonged drought, and to stimulate deeper root growth, irrigation was kept to a minimum sufficient to prevent crop failure, with a last-minute irrigation of 15 mm on 11 July 2017 shortly before the second harvest. Plant growth was stunted across all treatments, with lower DM and N yields relative to the previous summer, except timothy which maintained N yield.

Clovers tolerated the summer drought in L2 well and had similar DM yields both years, whereas bluegrass and meadow fescue were the species most negatively affected by the drought, with a nearly 40% decrease relative to the summer yield in L1, despite a visually tight coverage (Fig. 4 A, Suppl. Table S 6 B).

There was an exceptionally heavy rain event of ~55 mm in August 2017 between the second and third harvests (Suppl. Fig. S 2, Wolff et al., 2018). The L2 autumn harvest of timothy was reduced by foliar disease.

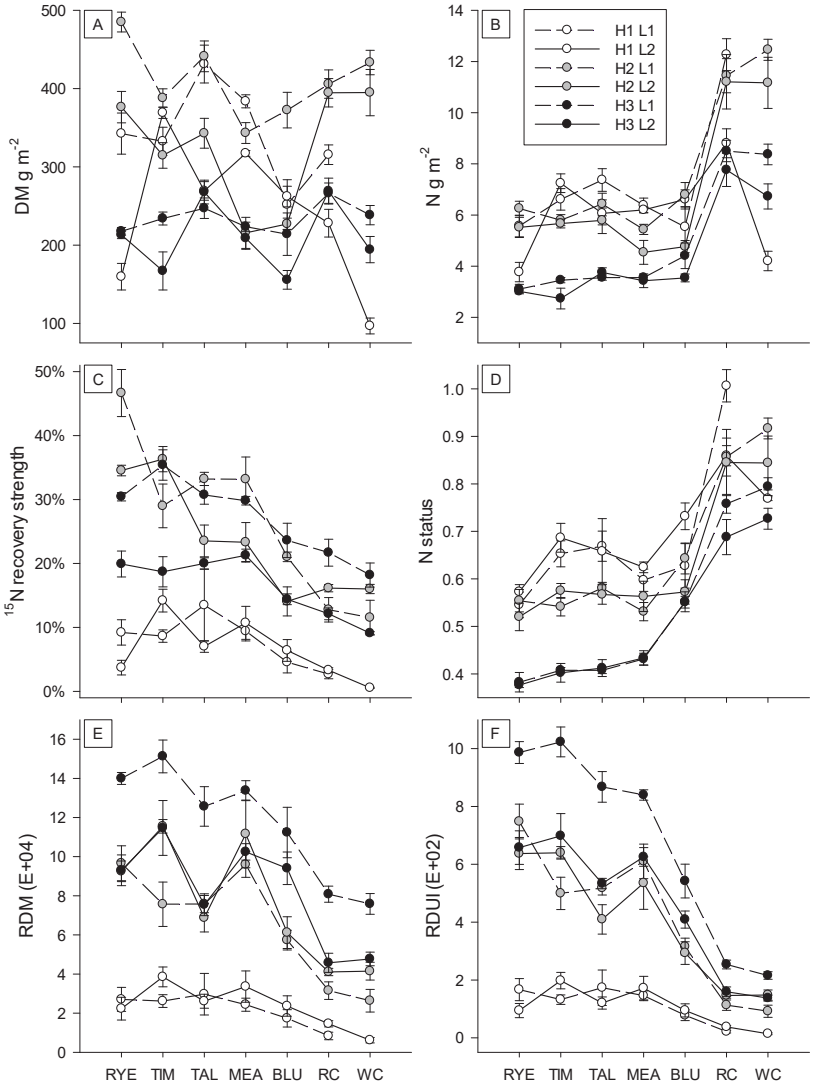


Figure 4: (A) **DM yield** (g DM m^{-2}), (B) **N yield** (g N m^{-2}) of H1 through H3 in L1 and L2. (C) **^{15}N recovery strength** (% recovered of label remaining in soil, Eqn. 3), (D) **N status** (1 indicates no deficiency, see 2.7. Plant nutritional N status), (E) **RDM** (^{15}N recovery strength per DM, Eqn. 5), and (F) **RDUI** (^{15}N recovery strength per N, Eqn. 4), Mean values of 4 replicates (±SE). A line connects results within each harvest for readability. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover (not harvested in spring of L1). See for *post hoc* Tukey groupings.

Table 2: Cumulative herbage **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}), and total **^{15}N herbage uptake** (mg ^{15}N m^{-2}) of three consecutive harvests after ^{15}N labeling in early spring 2016 (L1) and 2017 (L2), and related weighted ratios: **Recovered ^{15}N to N yield** (mg ^{15}N g^{-1} N), **Recovered ^{15}N to DM yield** (mg ^{15}N g^{-1} DM), and **N concentration in the DM** (g N 100 g^{-1} DM). Mean values of 4 replicates (±SE). Letters indicate *post hoc* Tukey groupings (α=0.05), analyzed separately for each labeling.

Cumulative total 3 harvests (±SE) Tukey α=0.05	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	^{15}N herbage uptake (mg ^{15}N m^{-2})	Recovered ^{15}N/N yield (mg ^{15}N g^{-1} N)	Recovered ^{15}N/DM yield (mg ^{15}N g^{-1} DM)	N conc. (g N 100 g^{-1} DM)
L1						
Per. Ryegrass	1045 (±30.0) AB	14.9 (±0.8) C	44.8 (±1.9) A	3.02 (±0.18) A	0.043 (±0.002) A	1.43 (±0.04) D
Timothy	955 (±33.4) BC	15.9 (±0.7) C	39.2 (±2.4) A	2.47 (±0.15) B	0.041 (±0.003) A	1.66 (±0.03) C
Tall fescue	1120 (±17.3) A	17.4 (±0.4) C	40.5 (±2.2) A	2.33 (±0.08) B	0.036 (±0.002) AB	1.55 (±0.04) CD
Meadow fescue	951 (±24.3) BC	15.4 (±0.5) C	38.8 (±2.0) A	2.53 (±0.10) AB	0.041 (±0.001) A	1.62 (±0.06) C
Bluegrass	839 (±52.2) C	16.7 (±1.2) C	28.8 (±1.0) B	1.75 (±0.14) C	0.035 (±0.002) AB	1.99 (±0.03) B
Red clover	988 (±26.0) AB	32.2 (±0.7) A	22.6 (±2.0) BC	0.70 (±0.06) D	0.023 (±0.002) C	3.26 (±0.03) A
White clover [a]	672 (±16.4) D	20.8 (±0.5) B	18.8 (±0.8) C	0.90 (±0.04) D	0.028 (±0.001) BC	3.10 (±0.03) A
L2						
Per. Ryegrass	749 (±28.1) BC	12.3 (±0.4) C	33.5 (±0.9) AB	2.73 (±0.11) A	0.045 (±0.002) A	1.64 (±0.02) C
Timothy	851 (±17.8) AB	15.7 (±0.6) C	37.6 (±1.5) A	2.42 (±0.14) AB	0.044 (±0.002) AB	1.84 (±0.05) C
Tall fescue	880 (±27.9) AB	15.6 (±1.0) C	29.2 (±0.9) B	1.90 (±0.17) BC	0.033 (±0.002) BCD	1.78 (±0.12) C
Meadow fescue	742 (±24.6) BC	14.2 (±0.7) C	31.0 (±2.6) AB	2.21 (±0.23) AB	0.042 (±0.004) ABC	1.91 (±0.04) C
Bluegrass	645 (±8.5) C	14.9 (±0.4) C	21.0 (±2.2) C	1.41 (±0.14) C	0.032 (±0.003) CD	2.31 (±0.06) B
Red clover	892 (±49.8) A	27.8 (±2.2) A	19.5 (±0.5) C	0.71 (±0.05) D	0.022 (±0.001) D	3.10 (±0.07) A
White clover	686 (±39.2) C	22.1 (±1.3) B	16.3 (±0.6) C	0.75 (±0.07) D	0.024 (±0.002) D	3.22 (±0.03) A

[a] White clover was not harvested the first cut of L1.

All species except white clover had lower annual DM yields in the third production year (L2) compared to the second production year (L1) (Table 2). The N yield was more similar between the two years, and on average the grasses yielded about 16 and 14.5 g N m^{-2}, corresponding to 80% and 72% of the fertilizer N applied in L1 and L2, respectively (Table 2). The N yield of red clover was about twice as much as that of the grasses (~30 g N m^{-2}), and higher than that of white clover (~21 g N m^{-2}), reflecting a higher DM yield rather than differences in N concentration.

The herbage uptake of deep-placed ^{15}N was smaller in L2 than in L1 (See 3.5. Seasonal variations for differences within each season). The tall-growing grasses recovered the most ^{15}N annually both years (~60% of applied in L1 and 50% of applied in L2), whereas the clovers recovered about half as much (Table 2). Bluegrass recovered similar or slightly higher amounts of ^{15}N as the clovers; it also had markedly higher N concentration but lower ^{15}N N^{-1} than the other grasses (Table 2). Tall fescue was the highest DM-yielding species both years; however, despite being reputed as deep rooting, it did not recover more ^{15}N than the other tall-growing grasses, and it tended to have lower ^{15}N DM^{-1} and ^{15}N N^{-1} than the other tall-growing grasses (Table 2). Ryegrass was among the species recovering the most ^{15}N both years, despite its poor spring regrowth. Timothy recovered more ^{15}N relative to the other tall-growing grasses in L2 compared to L1, corresponding with its good winter survival and persistence.

3.5. Seasonal variations

There was a strong seasonality in DM yield, N status, and ^{15}N recovery strength (Fig. 4). The general decrease in annual DM from L1 to L2 was reflected in all 3 harvests. Though there were exceptions pertaining to winter survival and drought response of some species (See 3.4: Growth conditions and plant performance), the DM yields of the spring and summer harvests were otherwise comparable in size both years. Autumn DM yields were ~20-40% lower, and the tall-growing grasses were particularly N-deficient in autumn of both years (Fig. 4 A, D). The N status (See 2.7. Plant nutritional N status) of each grass species was similar at each harvest in L2 as in the corresponding harvest in L1, with the exception that bluegrass was more N deficient in spring of L2 than in spring of L1 (Fig. 4 D). N status of the clovers was higher than that of the grasses, though was also somewhat reduced in the autumn, and in L2 compared to L1.

The ^{15}N recovery strength also varied strongly between harvests: in both years it was weakest in spring and stronger in the summer and autumn harvests (Fig. 4 C). In the grasses, ^{15}N recovery strength ranged from 4%-14% in the spring, markedly lower than in the summer (14%-47%) and autumn harvests (14%-35%). In the autumn of L2 all species had much lower ^{15}N recovery than in L1, even though the autumn N yield was as high in L2 as in L1 (Fig. 4 B, C).

The order of tall-growing grass species with the strongest ^{15}N recovery shifted throughout each harvest (Fig. 4 C; see Suppl. Table S 6 A for *post hoc* Tukey grouping). In the spring harvest of L2 timothy recovered the most ^{15}N together with meadow fescue, while there were no significant species effects in the spring harvest of L1. In the summer harvests ryegrass had the highest ^{15}N recovery strength both years, a position shared with timothy in summer of L2. In the autumn harvests there were no marked differences between the tall grasses, although timothy tended to have the highest ^{15}N recovery strength in L1 and meadow fescue showed a similar tendency in L2. Tall fescue did not have the highest ^{15}N recovery strength in any harvest. The ^{15}N recovery of bluegrass was generally between that of grasses and clovers, though in the drought summer bluegrass did not have better N status than the tall-growing grasses and had lower ^{15}N recovery strength than the clovers (Fig. 4 C, D).

The Relative Deep Uptake Index (RDUI), which is ^{15}N recovery strength relative to N yield (Eqn. 4, Fig. 4 F), was the same for a given species in both springs and in both summers, but not in both autumn harvests. RDUI was highest across all species in autumn of L1, however, in autumn of L2 RDUI was only slightly higher than in the summer, despite similar DM yields and N status as in the previous autumn (Fig. 4 A, D, F). RDUI was strongest in the tall-growing grasses, followed by bluegrass which consistently had RDUI halfway between tall-growing grasses and the clovers, which reached only 15-20% of the maximum RDUI observed in the tall-growing grasses at each harvest (Suppl. Table S 6).

The Recovery per Dry Matter (RDM) weights the ^{15}N recovery strength by DM yield (Eqn. 5). RDM followed much the same trends as RDUI (Fig. 4 E, F), and in both autumns and the drought summer of L2, highlights weak ^{15}N recovery by tall fescue and strong

^{15}N recovery by timothy. There was no significant effect of species on RDM in the spring harvest of L1.

3.6. Relationship between ^{15}N recovery strength and other performance variables

We hypothesized that the ^{15}N recovery strength would increase with plant vigor, as indicated by the DM yield. As seen in Fig. 4 (A, C) this was clearly not the case across harvests, as the DM yield of the spring harvest was roughly similar to that in the summer, while the ^{15}N recovery strength was much lower. It was also not true for the clovers compared to the grasses.

However, plant vigor could be important within a growing period. We checked this within each seasonal period (spring, summer or autumn, each group including L1 and L2) and separately for grasses (5 species) and clovers (2 species) by comparing the correlation of ^{15}N recovery strength with DM yield and other variables. Overall, DM yield predicted ^{15}N recovery strength significantly and better than did N yield, which in turn was a better predictor of ^{15}N recovery strength than N concentration in the herbage, especially in grasses (Suppl. Table S 7). However, an analysis of the scatter plots showed that the relationships were not linear, and the pattern changed between harvests (data not shown).

We also hypothesized that N deficiency would increase uptake of deep-placed ^{15}N. At a superordinate level the ^{15}N recovery strength and the N status changed in reverse order going from the clovers through bluegrass to the tall-growing grasses; similarly, an increasing N deficiency from spring to autumn corresponded to increasing ^{15}N recovery strength (Fig. 4). However, N status was not a good predictor of ^{15}N recovery strength within seasonal periods for the grasses or the clovers (Suppl. Table S 7), and it did not correlate any better with ^{15}N recovery strength than did herbage N concentration. There is necessarily a dependency between N status and plant vigor, which, as seen, was important for ^{15}N uptake.

The best explanatory variable for ^{15}N recovery strength within a harvest period and group of species was RDUI (r ranging 0.75-0.95), in close competition with RDM, (r ranging 0.64-0.96, Suppl. Table S 7). Also in the fourth through sixth harvests in the second year after labeling, ^{15}N recovery strength was clearly correlated with RDUI and

RDM. This indicates that relative preference and specific root affinity for deep NH_4^+ was more important than plant vigor (DM yield) alone.

4. Discussion

4.1. Clinoptilolite as deep $^{15}NH_4^+$ reservoir over successive harvests

Most ^{15}N studies on root uptake of N in grassland species either injected or buried quick-dispersing solutions of $^{15}NO_3^-$ (Kristensen and Thorup-Kristensen, 2004), $^{15}NH_4^+$ (Pirhofer-Walzl et al., 2013), double-labeled NH_4NO_3 (Hoekstra et al., 2015), or ^{15}N-enriched urea (Malcolm et al., 2015). These studies either harvested the plants once and shortly after injection (6 days - Kristensen and Thorup-Kristensen, 2004; 10 days - Pirhofer-Walzl et al., 2013; up to 3-4 weeks – Hoekstra et al., 2015), or used multiple applications over a longer study period with multiple harvests as Malcolm et al. (2015). In a unique approach, von Felten et al. (2012) used enriched ^{15}N plant litter as slow-release ^{15}N source in pots and harvested plants up to 11 months later. Otherwise, we are not aware of previous experiments following the recovery of ^{15}N over several subsequent harvests after a single application.

Our method of using $^{15}NH_4^+$ adsorbed to clinoptilolite is thus a new and complementary tool for the study of root activity, with several advantages. We obtained a high level of ^{15}N label tracing up to 17 months after application (Suppl. Table S 5). The fact that one and five months after application the passive upward diffusion of ^{15}N in absence of vegetation was negligible (Fig. 2 A) supports that the ^{15}N measured in herbage was a real effect of deep (~40 cm) root activity in this naturally compact silty clay soil (bulk density 1.54 g cm^{-3}). Where we obtained evidence of $^{15}NH_4^+$ uptake, we can assume the deep roots would be able recover leached NO_3^- even better, due to its higher diffusion and thus likelihood of being transported to the root surface.

Only 8-15% of the applied ^{15}N was unaccounted for in our tall-growing grass subplots after 17 months (Fig. 3). Our estimation accounts for ^{15}N contained in fine roots or remineralized from plant litter present in the soil analyzed, but excludes ^{15}N stored in larger roots, crowns and stubble left after harvesting, (height 5 cm) or still adsorbed to

the clinoptilolite, which we did not have the opportunity to analyze. Including them would probably raise the level of recovery even higher (Davis et al., 2006).

The fact that after 17 months we only detected 1-4% of the ^{15}N applied to planted subplots at the 46-60 cm soil depth, below the clinoptilolite, and with no significant differences between species (Fig. 2 B, C) suggests little loss by diffusion and leaching of NH_4^+ in this clayey soil. Since at most 2% of the original ^{15}N label was found in the subplot margins (See 2.4. ^{15N} recovery by plants and redistribution in the soil, Suppl. Fig. S 1), we conclude that the harvested area was sufficiently large to capture the plants' ability to recover the original label ("negative discard," Powlson and Barraclough, 1993, Kristensen and Thorup-Kristensen, 2004). The fact that margins of grass subplots contained some ^{15}N enrichment, while virtually no ^{15}N was detected in the margin of red clover, can be due to contribution by shedding in the grass subplots. Interestingly, there was no indication of horizontal ^{15}N transport outside the subplot through stolons in white clover and rhizomes in bluegrass.

The long study period allowed us to track active ^{15}N uptake from depth and its redistribution to the upper soil after two growing seasons, mostly to the upper 10 cm, likely through shedding of roots and aboveground litter.

4.2 Increased depth of root activity during the growing season

We found that high herbage production was necessary but not sufficient for efficient utilization of $NH4^+$ from the very compact and SOM-poor deep soil, below a well-structured and SOM-rich ploughed layer and dense root zone.

The slow-release ^{15}N label revealed little deep root activity during spring regrowth compared to summer and autumn, in which 2-3 times more ^{15}N was taken up in plant herbage. In grasses tiller death during the winter entails root death. In early spring, root regrowth is dominated by thick nodal roots, while secondary roots, which are thinner and more effective for nutrient capture, start growing later (Chen et al., 2016). Thus roots from new tillers take time to penetrate deeper soil in spring. This was best exemplified by ryegrass in L2, which had low but evenly-distributed winter tiller survival, followed by pronounced tillering in early spring and low ^{15}N recovery in the spring harvest, but high ^{15}N recovery in the summer. Also white clover and even red

clover, which under the field conditions of the experiment lost all green aboveground organs under snow cover, recovered very little ^{15}N in the spring compared to the summer and autumn harvests. This might indicate that shedding of active fine roots during winter and progressive active root depth during the growing season occur both in monocot and dicot species, as also observed by von Felten et al. (2012). Upon spring thaw, frost-sensitive and N-rich clover biomass from the previous autumn may also provide mineral N, perhaps in excess of immediate clover N needs (Byers et al., 2021; Sturite et al., 2021).

Kristensen and Thorup-Kristensen (2004) found a linear relationship between root density and ^{15}N recovery from different depths. Husse et al. (2017) found that from H2 to H4 of a six-cut grassland in Switzerland, shallow-rooting perennial ryegrass and white clover pure doubled their relative deep (30 cm) versus deep+shallow (3 cm) N uptake (similar to our RDUI), while deep-rooting chicory and red clover already achieved relatively deep uptake in H2, increasing it only slightly for H4. In our study, an increasing N deficiency from the first to the last harvest may also have stimulated deep root uptake; however, ^{15}N recovery increased much more than N deficiency, indicating that deep rooting was the most important mechanism for the increased recovery of deep-placed ^{15}N as the growing season proceeded.

That ^{15}N recovery strength often remained constant or decreased from the second to the third harvest may be partly because we did not account for ^{15}N stored in stubble and large roots. This likely overestimated the amount of ^{15}N remaining in the soil after H2 and thus underestimated the recovery strength of deep-placed ^{15}N at H3. It is also possible that the heavy rain event during the third regrowth period of L2 caused N leaching and/or plant stress, helping to explain the systematically lower ^{15}N recovery in autumn of L2 compared to autumn of L1.

An important conclusion is that approaches to studying deep nutrient uptake must consider that expression of this functional trait is seasonal (Chen et al., 2016). For example, an experiment on deep-placed ^{15}N recovery performed in the spring cannot be used to deduce the recovery of leached N in the autumn.

4.3 Species performance

In general and over the whole growing season, the tall-growing grasses exploited deep N equally well, except for tall fescue which had weakened ^{15}N recovery in the drought-affected L2. Conversely, the clovers took up the least deep N, without significant differences between red and white clover, which may indicate that red clover's persistent taproot is not advantageous for uptake of NH_4^+ from depth. High-yielding red clover tended nonetheless to recover slightly more ^{15}N label than low-yielding white clover. Furthermore, after two growing seasons the tall-growing grasses maintained a larger amount of ^{15}N in the plant-soil system than bluegrass and the clovers (Fig. 3), and in both springs the soil profile below the clovers contained twice as much mineral N as the grasses (Suppl. Table S 3). These major differences between groups of species was expected because of the thinner root system of the grasses compared with that of clovers, and the shallow rooting depth of bluegrass.

Although tall fescue is known for drought tolerance due to deep rooting, it tended to have lower uptake of ^{15}N relative to DM and N yield than other tall grasses, also under drought conditions. This agrees with the observation by Maire et al. (2009) that tall fescue has thick roots adapted for water transport but with low specific root area and low affinity for NH_4^+, and by Malcolm et al. (2015) that for tall fescue, growth vigor is more important than presence of deep roots for recovery of N from deeper soil, and dry conditions exacerbate its low affinity for NH_4^+.

Although ryegrass had poor winter survival, the surviving tillers demonstrated high plasticity and ability for vigorous growth in agreement with observations by Hoekstra et al. (2014), high N uptake and high yields per N uptake. The thin root system of ryegrass may also be advantageous for NH_4^+ uptake generally. Ryegrass utilized deep N as well or better than other tall-growing grasses, as exemplified in the summer harvest of L1, when it had the highest ^{15}N recovery strength (47%) observed throughout the experiment, in agreement with observations by Pirhofer-Walzl et al. (2013), and with observations that ryegrass has high affinity for NH_4^+ (Maire et al., 2009). This contrasts with our expectations and with the common perception of ryegrass as shallow-rooting (Husse et al., 2017), particularly when compared to chicory (Pirhofer-Walzl et al. 2013,

Hoekstra et al. 2015). However, we did not find studies comparing deep root activity of perennial ryegrass with that of tall fescue or other grasses.

Timothy in particular, and also meadow fescue displayed their well-known good winter hardiness (Østrem et al., 2015; Schjelderup et al., 1994) and performed well, with good utilization of deep N per DM and N yields, also under severe summer drought. Maire et al. (2009) observed that timothy has a weaker affinity for NH_4^+ than perennial ryegrass but a stronger affinity for NO_3^- and NH_4^+ than tall fescue.

As expected, bluegrass utilized less deep N than the other grasses due to both low DM yields and low $^{15}N\ DM^{-1}$. Considering that bluegrass had N yields m^{-2} as high as those of other grasses with higher DM yields, this confirms that bluegrass utilized shallower soil to satisfy its N requirements than the tall-growing grasses tested.

Both red and white clover utilized relatively less deep N than the other species tested, as we might expect due to the contribution of biological nitrogen fixation to their total N acquisition. Still, the clovers' strong recovery of deep-placed ^{15}N in summer and autumn demonstrates that deep N uptake is a part of their N acquisition strategy. Red clover recovered more ^{15}N than white clover due to its higher DM yields rather than to a higher $^{15}N\ DM^{-1}$; in this experiment red clover did not exploit its persistent taproot for deep NH_4^+ uptake. Furthermore, in the drought-affected summer harvest of L2 red clover had much weaker ^{15}N recovery than perennial ryegrass, despite similar yields, in contrast with findings by (Hoekstra et al., 2015) in a first-year sward.

Weighting ^{15}N uptake in herbage by the amount presumably left in the soil (^{15}N recovery strength), and by DM and N yields (RDM and RDUI), helped to identify differences in species' inherent ability to utilize NH_4^+ from deeper soil (Fig. 4 E, F). RDM highlights the relatively weak ^{15}N recovery by tall fescue relative to timothy, and to a lesser extent meadow fescue, during the drought summer and the autumns. Variations in growth vigor between these species appeared to affect neither the inherent ability to utilize deep N nor its utilization in proportion to total N uptake (RDUI, Fig. 4 A, E, F).

RDM and RDUI were markedly lower in autumn of L2 compared to L1, yet showed a similar species effect. It is possible that N deficiency caused by the preceding drought, stunting growth equally in the grasses, caused plants to reduce deep root activity in

favor of overcompensated aboveground growth when soil moisture returned (analogous to Hofer et al., 2017), or that less N_{min} was transported downward in the soil profile during the drought. If drought in L2 had reduced photosynthesis more than N uptake, we would have expected higher N status in grasses than in the summer harvest of the first year, but this was not the case.

Presumably, moderate N deficiency stimulated utilization of the N_{min} in the soil, explaining the relatively strong ^{15}N recovery in the N-deficient autumns despite low yields, but in this experiment, N status as such was not a variable useful for predicting recovery strength of deep-placed $^{15}NH_4^+$. Within individual summer or autumn harvests, some tall-growing grass species showed weak correlations between N status and deep N utilization, but N status did not vary enough to provide conclusive results. To better explore the effect of N status on deep N utilization by individual species, various levels of N deficiency should be induced under otherwise similar growing conditions.

4.4 Conclusions

Our slow-release method of using deep-placed $^{15}NH_4^+$ adsorbed to clinoptilolite allowed studying the combined influences of growing conditions and species effects on root uptake behavior in several regrowth periods (Hypothesis 1). This increases the chance of capturing interesting events such as a drought, and changes in root activity during the growing season.

Contrary to Hypothesis 2, ^{15}N recovery by grasses and clovers was small in the spring harvest, despite high growth vigor. This suggests that deep root activity takes time to re-establish after winter for both the monocot and dicot species studied. Both may shed active fine roots over winter; new tiller formation by grasses may delay deep root activity, and red clover's persistent taproot did not appear to be advantageous for spring ^{15}N recovery.

Also contrary to Hypothesis 2, recovery of deep-placed $^{15}NH_4^+$ was as strong or stronger in autumn than in the summer, despite lower yields. N deficient conditions in autumn correlated with stronger ^{15}N recovery relative to the summer. However, we lack evidence of an effect of N status on ^{15}N recovery strength by individual species and thus cannot conclude that N deficiency enhanced ^{15}N uptake (Hypothesis 3).

Our Hypothesis 4 that species in pure stands would exploit deep-placed $^{15}NH_4^+$ in the order: tall fescue > meadow fescue and timothy > perennial ryegrass > red clover > bluegrass > white clover was only confirmed for the groups (tall-growing grasses > bluegrass > clovers). Tall fescue was somewhat less efficient in taking up deep N than expected from its DM yield, likely due to thick roots with low affinity for NH_4^+.

Contrary to Hypothesis 5, ^{15}N recovery did not strongly decline in ryegrass in the second year. Ryegrass compensated for poor winter survival with vigorous regrowth, after which it was among the highest ^{15}N-recovering species, also under severe summer drought.

The fact that timothy and meadow fescue are equally capable of recovering N from deeper soil as ryegrass and tall fescue is interesting because these species survive and persist well in hemiboreal conditions, and with N yields equivalent to those of perennial ryegrass and better feeding quality than tall fescue. This confirms that they can be good strategic choices also for N utilization throughout the soil profile.

References

Baadshaug, O.H., Lantinga, E.A., 2002. ENGNOR, a Grassland Crop Growth Model for High Latitudes. Technical Documentation. (Reports from AUN. Department of Plant Sciences No. 2/2002). Agricultural University of Norway.

Bélanger, G., Gastal, F., 2000. Nitrogen utilization by forage grasses. Canadian Journal of Plant Science 80, 11–20. https://doi.org/10.4141/P99-033

Bleken, M.A., Rittl, T.F., Nadeem, S., Hansen, S., 2022. Roots and other residues from leys with or without red clover: Quality and effects on N_2O emission factors in a partly frozen soil following autumn ploughing. Science of The Total Environment 831, 154582. https://doi.org/10.1016/j.scitotenv.2022.154582

Byers, E., Bleken, M.A., Dörsch, P., 2021. Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH. Environ. Res. Commun. 3, 015001. https://doi.org/10.1088/2515-7620/abd623

Byers, E., Dörsch, P., Eich-Greatorex, S., Bleken, M.A. (2023) Deep N acquisition in hemiboreal cultivated grasslands: II. Niche and overyielding effects in mixtures. Manuscript II of this Thesis. Norwegian University of Life Sciences.

Cahill, J.F., McNickle, G.G., 2011. The Behavioral Ecology of Nutrient Foraging by Plants. Annual Review of Ecology, Evolution, and Systematics 42, 289–311. https://doi.org/10.1146/annurev-ecolsys-102710-145006

Chen, S.-M., Lin, S., Loges, R., Reinsch, T., Hasler, M., Taube, F., 2016. Independence of seasonal patterns of root functional traits and rooting strategy of a grass-clover sward from sward age and slurry application. Grass Forage Sci 71, 607–621. https://doi.org/10.1111/gfs.12222

Craine, J.M., Froehle, J., Tilman, D.G., Wedin, D.A., Chapin, III, F.S., 2001. The relationships among root and leaf traits of 76 grassland species and relative abundance along fertility and disturbance gradients. Oikos 93, 274–285. https://doi.org/10.1034/j.1600-0706.2001.930210.x

Davis, J.H., Griffith, S.M., Horwath, W.R., Steiner, J.J., Myrold, D.D., 2006. Fate of Nitrogen-15 in a Perennial Ryegrass Seed Field and Herbaceous Riparian Area. Soil Sci. Soc. Am. J. 70, 909–919. https://doi.org/10.2136/sssaj2005.0223

Ergon, Å., Kirwan, L., Bleken, M.A., Skjelvåg, A.O., Collins, R.P., Rognli, O.A., 2016. Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. Grass and Forage Science 71, 667–682. https://doi.org/10.1111/gfs.12250

Finn, J.A., Kirwan, L., Connolly, J., Sebastià, M.T., Helgadottir, A., Baadshaug, O.H., Bélanger, G., Black, A., Brophy, C., Collins, R.P., Čop, J., Dalmannsdóttir, S., Delgado, I., Elgersma, A., Fothergill, M., Frankow-Lindberg, B.E., Ghesquiere, A., Golinska, B., Golinski, P., Grieu, P., Gustavsson, A.-M., Höglind, M., Huguenin-Elie, O., Jørgensen, M., Kadziuliene, Z., Kurki, P., Llurba, R., Lunnan, T., Porqueddu, C.,

Suter, M., Thumm, U., Lüscher, A., 2013. Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. Journal of Applied Ecology 50, 365–375. https://doi.org/10.1111/1365-2664.12041

Großkinsky, D.K., Svensgaard, J., Christensen, S., Roitsch, T., 2015. Plant phenomics and the need for physiological phenotyping across scales to narrow the genotype-to-phenotype knowledge gap. EXBOTJ 66, 5429–5440. https://doi.org/10.1093/jxb/erv345

Hernandez, P., Picon-Cochard, C., 2016. Presence of Trifolium repens Promotes Complementarity of Water Use and N Facilitation in Diverse Grass Mixtures. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.00538

Hodge, A., 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytologist 162, 9–24. https://doi.org/10.1111/j.1469-8137.2004.01015.x

Hoekstra, N.J., Finn, J.A., Hofer, D., Lüscher, A., 2014. The effect of drought and interspecific interactions on depth of water uptake in deep- and shallow-rooting grassland species as determined by $\delta\ ^{18}O$ natural abundance. Biogeosciences 11, 4493–4506. https://doi.org/10.5194/bg-11-4493-2014

Hoekstra, N.J., Suter, M., Finn, J.A., Husse, S., Lüscher, A., 2015. Do belowground vertical niche differences between deep- and shallow-rooted species enhance resource uptake and drought resistance in grassland mixtures? Plant and Soil 394, 21–34. https://doi.org/10.1007/s11104-014-2352-x

Hofer, D., Suter, M., Buchmann, N., Lüscher, A., 2017. Nitrogen status of functionally different forage species explains resistance to severe drought and post-drought overcompensation. Agriculture, Ecosystems & Environment 236, 312–322. https://doi.org/10.1016/j.agee.2016.11.022

Husse, S., Lüscher, A., Buchmann, N., Hoekstra, N.J., Huguenin-Elie, O., 2017. Effects of mixing forage species contrasting in vertical and temporal nutrient capture on nutrient yields and fertilizer recovery in productive grasslands. Plant Soil 420, 505–521. https://doi.org/10.1007/s11104-017-3372-0

IUSS Working Group WRB, 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps, World Soil Resources Reports No. 106. FAO, Rome.

Jumpponen, A., Högberg, P., Huss-Danell, K., Mulder, C.P.H., 2002. Interspecific and spatial differences in nitrogen uptake in monocultures and two-species mixtures in north European grasslands: *Nitrogen uptake in north European grasslands*. Functional Ecology 16, 454–461. https://doi.org/10.1046/j.1365-2435.2002.00642.x

Kristensen, H.L., Thorup-Kristensen, K., 2004. Root Growth and Nitrate Uptake of Three Different Catch Crops in Deep Soil Layers. Soil Science Society of America Journal 68, 529. https://doi.org/10.2136/sssaj2004.0529

Lemaire, G., Salette, J., Sigogne, M., Terrasson, J.-P., 1984. Relation entre dynamique de croissance et dynamique de prélèvement d'azote pour un peuplement de graminées fourragères. I. — Etude de l'effet du milieu. Agronomie 4, 423–430. https://doi.org/10.1051/agro:19840503

Maire, V., Gross, N., da Silveira Pontes, L., Picon-Cochard, C., Soussana, J.-F., 2009. Trade-off between root nitrogen acquisition and shoot nitrogen utilization across 13 co-occurring pasture grass species. Functional Ecology 23, 668–679. https://doi.org/10.1111/j.1365-2435.2009.01557.x

Malcolm, B.J., Moir, J.L., Cameron, K.C., Di, H.J., Edwards, G.R., 2015. Influence of plant growth and root architecture of Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) on N recovery during winter. Grass and Forage Science 70, 600–610. https://doi.org/10.1111/gfs.12157

Mamolos, A.P., Veresoglou, D.S., 2000. Patterns of root activity and responses of species to nutrients in vegetation of fertile alluvial soil. Plant Ecology 148, 245–253. https://doi.org/10.1023/A:1009890630391

Milovanović, J., Eich-Greatorex, S., Krogstad, T., Rakic, V., Rajic, N., 2015. The use in grass production of clinoptilolite as an ammonia adsorbent and a nitrogen carrier. Journal of the Serbian Chemical Society 80, 1203–1214. https://doi.org/10.2298/JSC150317042M

Mommer, L., Van Ruijven, J., De Caluwe, H., Smit-Tiekstra, A.E., Wagemaker, C.A.M., Joop Ouborg, N., Bögemann, G.M., Van Der Weerden, G.M., Berendse, F., De Kroon, H., 2010. Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species: Below-ground species distributions in a biodiversity experiment. Journal of Ecology 98, 1117–1127. https://doi.org/10.1111/j.1365-2745.2010.01702.x

Murphy, P.N.C., O'Connell, K., Watson, S., Watson, C.J., Humphreys, J., 2013. Seasonality of nitrogen uptake, apparent recovery of fertilizer nitrogen and background nitrogen supply in two Irish grassland soils. Irish Journal of Agricultural and Food Research 52, 17–38.

Østrem, L., Rapacz, M., Larsen, A., Dalmannsdottir, S., Jørgensen, M., 2015. Influences of growth cessation and photoacclimation on winter survival of non-native Lolium–Festuca grasses in high-latitude regions. Environmental and Experimental Botany 111, 21–31. https://doi.org/10.1016/j.envexpbot.2014.10.008

Pirhofer-Walzl, K., Eriksen, J., Rasmussen, J., Høgh-Jensen, H., Søegaard, K., Rasmussen, J., 2013. Effect of four plant species on soil ^{15}N-access and herbage yield in temporary agricultural grasslands. Plant and Soil 371, 313–325. https://doi.org/10.1007/s11104-013-1694-0

Powlson, D.S., Barraclough, D., 1993. Mineralization and Assimilation in Soil–Plant Systems, in: Nitrogen Isotope Techniques. Elsevier, pp. 209–242. https://doi.org/10.1016/B978-0-08-092407-6.50013-4

Ravenek, J.M., Bessler, H., Engels, C., Scherer-Lorenzen, M., Gessler, A., Gockele, A., De Luca, E., Temperton, V.M., Ebeling, A., Roscher, C., Schmid, B., Weisser, W.W., Wirth, C., de Kroon, H., Weigelt, A., Mommer, L., 2014. Long-term study of root biomass in a biodiversity experiment reveals shifts in diversity effects over time. Oikos 123, 1528–1536. https://doi.org/10.1111/oik.01502

Schjelderup, I., Aastveit, A.H., Aastveit, K., 1994. Winter hardiness in marginal populations of timothy, in: Rognli, O.A., Solberg, E., Schjelderup, I. (Eds.), Breeding Fodder Crops for Marginal Conditions, Developments in Plant Breeding. Springer Netherlands, Dordrecht, pp. 61–68. https://doi.org/10.1007/978-94-011-0966-6_6

Sturite, I., Rivedal, S., Dörsch, P., 2021. Clover increases N_2O emissions in boreal leys during winter. Soil Biology and Biochemistry 163, 108459. https://doi.org/10.1016/j.soilbio.2021.108459

von Felten, S., Niklaus, P.A., Scherer-Lorenzen, M., Hector, A., Buchmann, N., 2012. Do grassland plant communities profit from N partitioning by soil depth? Ecology 93, 2386–2396. https://doi.org/10.1890/11-1439.1

von Felten, S., Schmid, B., 2008. Complementarity among species in horizontal versus vertical rooting space. Journal of Plant Ecology 1, 33–41. https://doi.org/10.1093/jpe/rtm006

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2021. Meteorologiske data for Ås 2020. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2018. Meteorologiske data for Ås 2017. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2017. Meteorologiske data for Ås 2016. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2016. Meteorologiske data for Ås 2015. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Deep N acquisition in hemiboreal cultivated grasslands: I. Species in pure stands

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina A. Bleken

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Supplementary materials

Table S 1: Soil sampling dates and depths for ^{15}N analysis, and corresponding ^{15}N natural abundance (AT% (±SE)) and soil bulk density. Note: soil samples taken 1 month after labeling from de-vegetated plots had different depth profiles than those taken at 5 months and those taken from pure stand plots (See 2.4. ^{15}N recovery by plants and redistribution in the soil).

100 cm^3 intact soil cores were taken from four plots (tall fescue, perennial ryegrass, and two mixture plots) and analyzed for bulk density. The top 10 cm of soil was removed; bulk density for this layer was taken from an earlier analysis in a nearby field (Bleken, unpublished). 4 cores were placed at 10-14 cm depth, then one core per ~7.5 cm depth in deeper soil layers down to 65 cm. Exact placements were noted and results were interpolated to match ^{15}N sampling depths; values shown in this table.

	Sampling date	Treatments	Depths	Natural Abundance ^{15}N AT%	BD g cm^{-3}
Experimental plots in L1: 17 months after labeling	One plot 26 September, remaining plots between 9 and 12 October 2017	All pure-stand experiment plots in L1	0-10 cm	0.37019 (±0.0003)	1.10
			10-24 cm	0.37019 (±0.0003)	1.29
			24-38 cm	0.36994	1.32
			38-46 cm	0.36986	1.50
			46-60 cm	0.36987	1.54
De-vegetated plots in L2: 1 month after labeling	24 May 2017	All 4 de-vegetated plots labeled in L2	0-20, 20-30, 30-38, 38-46, and 46-54 cm	Interpolated to fit different depths	Interpolated to fit different depths
De-vegetated plots in L2: 5 months after labeling	12 October 2017	All 4 de-vegetated plots labeled in L2	0-10, 10-24, 24-38, 38-46, and 46-60 cm	Same as L1	Same as L1

Table S 2: Soil sampling dates and depths for mineral N analysis.

Sampling date		Depths	Treatments	Extraction method
At time of Labeling 1 (L1)	28 April 2016	30-43 cm only	All 28 pure stand experiment plots in L1	45 g soil in 50 ml 2*M* KCl
End of 2016 season	26 October 2016	0-20, 20-40, and 40-60 cm	4 replicates each of similar plots from same field: all pure stand species.	25g soil in 50 ml 1*M* KCl
At time of Labeling 2 (L2)	19 April 2017	0-20, 20-30, and 30-43 cm	All 28 pure stand and 4 de-vegetated experiment plots in L2	25 g soil in 30 mL 1*M* KCl
End of 2017 season	30 Oct 2017	0-20, 20-40, and 40-60 cm	4 replicates each of similar plots from same field: perennial ryegrass, tall fescue, and red clover only.	30 g soil in 50 mL 1*M* KCl

Table S 3: Mineral N (sum of NO^{3-} and NH_4^+ N) by depth in soil **A)** removed when placing ^{15}N labeled clinoptilolite (which in L2 occurred before N fertilization on 2 May), and **B)** sampled at the end of the growing seasons L1 and L2. Means of 4 replicates (±SE). *Post hoc* Tukey grouping ($p<0.05$) for each sampling and date combination.

A) Early spring Min-N measurements at time of ^{15}N label placement

	Min-N g m^{-2}, 0-20 cm depth	Min-N g m^{-2}, 20-30 cm depth	Min-N g m^{-2}, 30-43 cm depth	Whole profile: 0-43 cm depth
L1: Sampled 28 April 2016				
Per. Ryegrass[1]	-	-	0.87 (±0.16) B	-
Timothy	-	-	1.02 (±0.13) B	-
Tall fescue	-	-	1.02 (±0.15) B	-
Meadow fescue	-	-	0.99 (±0.12) B	-
Bluegrass	-	-	0.82 (±0.04) B	-
Red clover	-	-	2.54 (±0.32) A	-
White clover	-	-	2.25 (±0.09) A	-
L2: Sampled 19 April 2017				
Per. Ryegrass	1.08 (±0.19) ABC	0.33 (±0.05) BC	0.55 (±0.05) B	1.96 (±0.16) B
Timothy	0.52 (±0.15) BC	0.37 (±0.06) BC	0.40 (±0.07) B	1.30 (±0.17) B
Tall fescue	0.54 (±0.12) BC	0.38 (±0.06) BC	0.33 (±0.03) B	1.25 (±0.10) B
Meadow fescue	0.77 (±0.19) BC	0.28 (±0.05) BC	0.42 (±0.02) B	1.48 (±0.20) B
Bluegrass	0.40 (±0.11) C	0.26 (±0.04) C	0.43 (±0.10) B	1.09 (±0.21) B
Red clover	1.54 (±0.39) AB	0.87 (±0.17) A	1.15 (±0.19) A	3.56 (±0.53) A
White clover	1.93 (±0.43) A	0.70 (±0.14) AB	1.24 (±0.12) A	3.87 (±0.34) A
De-vegetated	0.42 (±0.03) BC	0.32 (±0.08) BC	0.33 (±0.04) B	1.08 (±0.11) B

[1] Omits one soil sample in per. ryegrass, L1, with a Min-N value three times that of the other three replicates.

B) Autumn Min-N measurements at end of growing season after ^{15}N label placement

	Min-N g m^{-2}, 0-20 cm depth	Min-N g m^{-2}, 20-40 cm depth	Min-N g m^{-2}, 40-60 cm depth	Whole profile: 0-60 cm depth
L1: Sampled 26 October 2016				
Per. Ryegrass	0.38 (±0.03) C	0.38 (±0.04) BC	0.13 (±0.05) AB	0.89 (±0.07) C
Timothy	0.71 (±0.19) BC	0.46 (±0.05) BC	0.09 (±0.01) B	1.26 (±0.22) BC
Tall fescue	0.39 (±0.02) C	0.33 (±0.06) C	0.08 (±0.05) B	0.80 (±0.04) C
Meadow fescue	0.43 (±0.12) BC	0.30 (±0.05) C	0.11 (±0.03) AB	0.83 (±0.09) C
Bluegrass	0.50 (±0.10) BC	0.35 (±0.04) BC	0.08 (±0.01) B	0.93 (±0.11) C
Red clover	1.10 (±0.21) AB	0.92 (±0.20) A	0.32 (±0.03) A	2.35 (±0.41) AB
White clover	1.58 (±0.24) A	0.78 (±0.12) AB	0.28 (±0.10) AB	2.63 (±0.41) A
L2: Sampled 30 October 2017				
Per. Ryegrass	0.67 (±0.04) B	0.30 (±0.04) B	0.16 (±0.02) B	1.13 (±0.06) B
Tall fescue	0.45 (±0.03) C	0.21 (±0.04) B	0.14 (±0.04) B	0.81 (±0.08) B
Red clover	1.40 (±0.06) A	1.04 (±0.11) A	0.72 (±0.17) A	3.15 (±0.33) A

Table S 4: ^{15}N recovered in soil (percent of original ^{15}N label placed; See Fig. 2 in article). Means of 4 replicates (±SE). One-sided t-tests had $p<0.05$ unless noted.

A) De-vegetated plots labeled in L2, 1 and 5 months after labeling

Depth	De-veg 1 mo.	Depth	De-veg 5 mo.
0-20 cm	0% (±0.4%), N.S.	**0-10 cm**	0% (±0.1%) [1]
20-30 cm	0% (±0.2%), p<0.1	**10-24 cm**	0% (±0.1%) [1]
30-38 cm	1% (±0.2%)	**24-38 cm**	2% (±0.4%)
38-46 cm	3% (±0.3%)	**38-46 cm**	4% (±1.0%)
46-54 cm	2% (±0.3%)	**46-60 cm**	7% (±1.3%)

[1] After 5 months, combined ^{15}N in ploughing depth (0-24 cm) was less than 1% ($p<0.05$).

B) Experimental plots: 17 months after Labeling L1

Depth	Per. Ryegrass[1]	Timothy	Tall fescue	Meadow fescue
0-10 cm	8% (±1.3%)	7% (±1.7%)	13% (±9.1%), p=0.12	8% (±1.5%)
10-24 cm	2% (±0.3%)	3% (±0.4%)	2% (±0.4%)	4% (±1.6%)
24-38 cm	4% (±1.8%)	2% (±0.4%)	2% (±0.4%)	8% (±2.7%)
38-46 cm	3% (±1.6%), p<0.1	3% (±0.8%)	2% (±1.0%), p<0.1	5% (±1.5%)
46-60 cm	3% (±1.4%), p<0.1	1% (±0.2%)	3% (±1.3%)	2% (±0.6%)

	Bluegrass	Red clover	White clover
0-10 cm	9% (±3.3%)	10% (±2.7%)	9% (±1.8%)
10-24 cm	2% (±0.5%)	8% (±3.7%), p<0.1	4% (±0.9%)
24-38 cm	2% (±0.7%)	4% (±0.6%)	3% (±0.5%)
38-46 cm	2% (±0.2%)	3% (±0.6%)	3% (±0.6%)
46-60 cm	1% (±0.6%), p<0.1	4% (±1.0%)	3% (±0.9%)

[1] The soil sample from one replicate of perennial ryegrass in autumn 2017 was removed from analysis because its ^{15}N enrichment was 38 times that of the three other replicates, indicating the sample likely contained ^{15}N-labeled clinoptilolite.

Table S 5: Results not presented in the article: herbage harvested in the second year after labeling in L1 (H4, H5, H6 in 2017) and L2 (H4 in 2018). **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}), **N concentration in the DM** (g N 100 g^{-1} DM), **N status** (1 indicates no deficiency, see 2.7. Plant nutritional N status), and **^{15}N recovery strength** (% recovery of label remaining in soil, Eqn. 3). Mean of 4 replicates (±SE).

	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	N conc. (% g N g^{-1} DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)
L1, H4: Harvested 6 June 2017, labeled 26-28 April 2016					
Per. Ryegrass	246.1 (±19.7)	4.7 (±0.4)	1.92% (±0.03%)	0.54 (±0.05)	4.0% (±0.4%)
Timothy	350.3 (±10.7)	7.5 (±0.4)	2.14% (±0.13%)	0.72 (±0.07)	18.0% (±1.3%)
Tall fescue	247.0 (±09.2)	5.0 (±0.1)	2.04% (±0.06%)	0.58 (±0.02)	10.0% (±1.1%)
Meadow fescue	307.4 (±08.8)	6.2 (±0.1)	2.03% (±0.01%)	0.64 (±0.01)	11.7% (±0.7%)
Bluegrass	238.5 (±17.6)	5.4 (±0.4)	2.26% (±0.02%)	0.63 (±0.05)	13.0% (±1.6%)
Red clover	205.4 (±06.6)	8.0 (±0.4)	3.89% (±0.09%)	0.83 (±0.05)	12.0% (±1.7%)
White clover	111.3 (±10.5)	4.8 (±0.4)	4.33% (±0.16%)	0.78 (±0.05)	3.3% (±0.2%)
L2, H4: Harvested 30 May 2018, labeled 18-20 April 2017					
Per. Ryegrass	173.8 (±09.6)	4.8 (±0.1)	2.79% (±0.08%)	0.69 (±0.02)	2.7% (±0.2%)
Timothy	350.3 (±22.7)	7.3 (±0.6)	2.09% (±0.09%)	0.71 (±0.09)	10.3% (±0.6%)
Tall fescue	309.2 (±10.6)	7.0 (±0.3)	2.27% (±0.05%)	0.72 (±0.04)	12.9% (±2.4%)
Meadow fescue	339.6 (±10.0)	7.3 (±0.2)	2.15% (±0.05%)	0.71 (±0.03)	10.9% (±0.7%)
Bluegrass	384.8 (±50.0)	10.0 (±1.4)	2.59% (±0.03%)	0.94 (±0.18)	15.4% (±1.2%)
Red clover	156.7 (±23.4)	5.4 (±0.9)	3.43% (±0.06%)	0.67 (±0.08)	5.3% (±0.9%)
White clover	86.1 (±11.7)	3.3 (±0.4)	3.81% (±0.03%)	0.66 (±0.02)	1.7% (±0.1%)

	DM Yield ($g\ DM\ m^{-2}$)	N yield ($g\ N\ m^{-2}$)	N conc. (% $g\ N\ g^{-1}$ DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)
L1, H5: Harvested 19 July 2017, labeled 26-28 April 2016					
Per. Ryegrass	266.7 (±14.0)	4.8 (±0.3)	1.81% (±0.02%)	0.53 (±0.03)	2.6% (±0.1%)
Timothy	243.2 (±07.4)	4.8 (±0.4)	1.98% (±0.10%)	0.55 (±0.07)	5.1% (±0.4%)
Tall fescue	250.6 (±28.8)	4.8 (±0.6)	1.91% (±0.06%)	0.55 (±0.07)	4.4% (±0.9%)
Meadow fescue	173.7 (±15.2)	3.7 (±0.4)	2.11% (±0.08%)	0.52 (±0.05)	3.1% (±0.4%)
Bluegrass	198.9 (±16.1)	4.1 (±0.3)	2.06% (±0.05%)	0.53 (±0.02)	4.2% (±0.5%)
Red clover	313.5 (±39.3)	9.0 (±1.1)	2.87% (±0.05%)	0.74 (±0.10)	7.6% (±1.0%)
White clover	271.8 (±27.9)	8.3 (±0.9)	3.05% (±0.05%)	0.73 (±0.09)	5.7% (±1.1%)

	DM Yield ($g\ DM\ m^{-2}$)	N yield ($g\ N\ m^{-2}$)	N conc. (% $g\ N\ g^{-1}$ DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)
L1, H6: Harvested 18 Sep. 2017, labeled 26-28 April 2016					
Per. Ryegrass	189.3 (±04.7)	2.9 (±0.1)	1.55% (±0.05%)	0.40 (±0.03)	1.9% (±0.2%)
Timothy	171.8 (±12.6)	2.9 (±0.2)	1.69% (±0.04%)	0.42 (±0.01)	3.3% (±0.5%)
Tall fescue	235.9 (±12.5)	3.4 (±0.1)	1.46% (±0.09%)	0.40 (±0.04)	2.4% (±0.3%)
Meadow fescue	190.6 (±02.9)	3.3 (±0.1)	1.71% (±0.05%)	0.44 (±0.03)	2.5% (±0.3%)
Bluegrass	179.4 (±01.2)	4.0 (±0.1)	2.23% (±0.08%)	0.56 (±0.04)	3.2% (±0.4%)
Red clover	239.8 (±10.9)	6.8 (±0.4)	2.82% (±0.04%)	0.64 (±0.04)	3.6% (±0.5%)
White clover	142.7 (±10.6)	5.4 (±0.4)	3.77% (±0.06%)	0.72 (±0.03)	2.1% (±0.3%)

Table S 6: Herbage **DM yield** (g DM m^{-2}) and **N yield** (g N m^{-2}) in each of the first three harvests after labeling in early spring 2016 (L1) and 2017 (L2). Also: **N concentration in the DM** (g N 100 g^{-1} DM), **N status** (1 indicates no deficiency, see 2.7. Plant nutritional N status), **^{15}N recovery strength** (percent recovered of label remaining in soil, Eqn. 3), **RDUI** (^{15}N recovery strength to N yield, Eqn. 4), and **RDM** (^{15}N recovery strength to DM yield, Eqn. 5). Mean values of 4 replicates (±SE). Letters indicate post hoc Tukey groupings (α=0.05) analyzed separately for each labeling and harvest. White clover was not harvested the first cut of L1.

A) Spring harvests, H1

	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	N conc. (% g N g^{-1} DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)	RDUI (E+02) (^{15}N recovery strength g^{-1} N m^2)	RDM (E+04) (^{15}N recovery strength g^{-1} DM m^2)
L1: Harvested 1 June 2016, labeled 26-28 April 2016							
Per. Ryegrass	343 (±26.3) ABC	5.6 (±0.4) B	1.63% (±0.05%) D	0.55 (±0.03) B [1]	9.2% (±2.0%) [2]	1.67 (±0.38) A	2.71 (±0.62) [2]
Timothy	333 (±17.9) BC	6.6 (±0.4) B	1.98% (±0.03%) C	0.65 (±0.03) B	8.6% (±1.0%)	1.32 (±0.16) AB	2.62 (±0.33)
Tall fescue	431 (±24.2) A	7.4 (±0.4) B	1.71% (±0.01%) D	0.67 (±0.03) B	13.5% (±5.6%)	1.73 (±0.61) A	2.97 (±1.06)
Mea. fescue	384 (±8.4) AB	6.4 (±0.3) B	1.66% (±0.05%) D	0.60 (±0.02) B	9.4% (±1.5%)	1.46 (±0.17) AB	2.43 (±0.34)
Bluegrass	252 (±31.7) C	5.5 (±0.8) B	2.19% (±0.05%) B	0.63 (±0.05) B	4.6% (±1.7%)	0.78 (±0.19) AB	1.74 (±0.44)
Red clover	316 (±12.5) BC	12.3 (±0.6) A	3.88% (±0.05%) A	1.01 (±0.03) A	2.7% (±0.7%)	0.21 (±0.05) B	0.84 (±0.19)
White clover	-	-	-	-	-	-	-
L2: Harvested 6 June 2017, labeled 18-20 April 2017							
Per. Ryegrass	160 (±17.0) D	3.8 (±0.4) D	2.37% (±0.03%) CD	0.57 (±0.02) C [3]	3.7% (±1.1%) C	0.94 (±0.25) AB	2.23 (±0.58) ABC
Timothy	369 (±7.2) A	7.2 (±0.4) AB	1.96% (±0.07%) D	0.69 (±0.03) BC	14.2% (±1.8%) A	1.98 (±0.29) A [4]	3.85 (±0.50) A
Tall fescue	269 (±11.8) BC	6.1 (±0.8) BC	2.23% (±0.18%) CD	0.66 (±0.07) BC	7.0% (±0.9%) BC	1.20 (±0.21) AB	2.61 (±0.36) ABC
Mea. fescue	317 (±3.6) AB	6.2 (±0.1) BC	1.96% (±0.03%) D	0.62 (±0.01) BC	10.7% (±2.6%) AB	1.72 (±0.41) A	3.36 (±0.80) AB
Bluegrass	262 (±13.2) BC	6.6 (±0.4) B	2.52% (±0.09%) C	0.73 (±0.03) AB	6.4% (±1.7%) BC	0.95 (±0.23) AB	2.36 (±0.52) ABC
Red clover	228 (±17.7) C	8.8 (±0.6) A	3.87% (±0.09%) B	0.86 (±0.02) A	3.3% (±0.4%) C	0.38 (±0.05) B [5]	1.46 (±0.16) BC
White clover	97 (±10.1) E	4.2 (±0.4) CD	4.37% (±0.07%) A	0.77 (±0.00) AB	0.6% (±0.1%) C	0.14 (±0.03) B	0.62 (±0.14) C

[1] Ryegrass was significantly more N-limited when tested versus timothy and tall fescue in L1 H1 ($p<0.05$). [2] Species effect on ^{15}N recovery strength and RDM was not significant in L1 H1 ($p=0.11$, 0.14). [3] Ryegrass was significantly more N-limited when tested versus timothy, meadow fescue and bluegrass in L2 H1 ($p<0.05$). [4] Timothy had significantly higher RDUI when tested versus ryegrass in L2 H1 ($p<0.05$). [5] Red clover had sig. higher RDUI versus white clover in L2 H1 ($p<0.05$).

B) Summer harvests, H2

	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	N conc. (% g N g^{-1} DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)	RDUI (E+02) (^{15}N recovery strength g^{-1} N m^2)	RDM (E+04) (^{15}N recovery strength g^{-1} DM m^2)
L1: Harvested 12 July 2016, labeled 26-28 April 2016							
Per. Ryegrass	485 (±12.7) A	6.3 (±0.3) B	1.29% (±0.05%) D	0.55 (±0.02) B	46.7% (±3.7%) A	7.48 (±0.60) A	9.66 (±0.89) A
Timothy	388 (±11.8) BC	5.8 (±0.2) B	1.50% (±0.06%) CD	0.54 (±0.02) B	29.0% (±3.4%) B	5.00 (±0.56) BC	7.57 (±1.13) AB
Tall fescue	441 (±19.7) AB	6.4 (±0.2) B	1.46% (±0.05%) CD	0.58 (±0.01) B	33.2% (±1.0%) B	5.18 (±0.24) B	7.58 (±0.44) AB
Mea. fescue	343 (±13.3) C	5.4 (±0.2) B	1.59% (±0.08%) BC	0.53 (±0.02) B	33.2% (±3.5%) B	6.10 (±0.60) AB	9.60 (±0.65) A
Bluegrass	373 (±22.7) BC	6.8 (±0.5) B	1.82% (±0.03%) B	0.64 (±0.03) B	21.0% (±0.7%) BC	3.16 (±0.29) C	5.73 (±0.50) BC
Red clover	406 (±18.1) BC	11.4 (±0.7) A	2.82% (±0.06%) A	0.86 (±0.04) A	12.8% (±1.9%) C	1.13 (±0.18) D	3.15 (±0.45) C
White clover	433 (±15.6) AB	12.5 (±0.4) A	2.88% (±0.02%) A	0.92 (±0.02) A	11.5% (±2.7%) C	0.92 (±0.21) D	2.64 (±0.58) C
L2: Harvested 19 July 2017, labeled 18-20 April 2017							
Per. Ryegrass	376 (±20.1) A	5.5 (±0.4) B	1.46% (±0.05%) C	0.52 (±0.03) B	34.5% (±0.8%) A	6.37 (±0.55) A	9.25 (±0.52) AB
Timothy	315 (±16.3) AB	5.7 (±0.2) B	1.81% (±0.08%) BC	0.57 (±0.02) B	36.3% (±2.0%) A	6.40 (±0.22) A	11.55 (±0.35) A
Tall fescue	343 (±19.1) A	5.8 (±0.2) B	1.70% (±0.10%) BC	0.57 (±0.02) B	23.5% (±2.5%) B	4.10 (±0.51) BC	6.88 (±0.73) BC
Mea. fescue	216 (±19.9) C	4.5 (±0.5) B	2.10% (±0.09%) B	0.56 (±0.03) B	23.3% (±3.1%) B	5.35 (±0.91) AB	11.16 (±1.73) A
Bluegrass	227 (±7.5) BC	4.8 (±0.2) B	2.10% (±0.10%) B	0.57 (±0.03) B	14.1% (±2.3%) C	2.93 (±0.39) CD	6.12 (±0.81) BC
Red clover	395 (±18.1) A	11.2 (±1.1) A	2.83% (±0.16%) A	0.85 (±0.07) A	16.1% (±0.6%) BC	1.46 (±0.09) D	4.11 (±0.19) C
White clover	395 (±29.5) A	11.2 (±1.0) A	2.82% (±0.05%) A	0.84 (±0.06) A	16.0% (±0.7%) BC	1.48 (±0.18) D	4.15 (±0.45) C

C) Autumn harvests, H3

	DM Yield ($g\ DM\ m^{-2}$)	N yield ($g\ N\ m^{-2}$)	N conc. (% $g\ N\ g^{-1}$ DM)	N status: (N conc. / N_{crit})	^{15}N recovery strength (% mg ^{15}N uptake mg^{-1} ^{15}N remaining in soil)	RDUI (E+02) (^{15}N recovery strength $g^{-1}\ N\ m^{2}$)	RDM (E+04) (^{15}N recovery strength $g^{-1}\ DM\ m^{2}$)
L1: Harvested 2 Sep 2016, labeled 26-28 April 2016							
Per. Ryegrass	218 (±4.9) [6]	3.1 (±0.2) B	1.43% (±0.07%) D	0.38 (±0.02) C	30.4% (±0.7%) AB	9.86 (±0.38) AB	14.00 (±0.30) AB
Timothy	234 (±8.3)	3.5 (±0.1) B	1.48% (±0.02%) D	0.41 (±0.01) C	35.4% (±2.4%) A	10.23 (±0.52) A	15.13 (±0.84) A
Tall fescue	247 (±13.1)	3.6 (±0.1) B	1.45% (±0.05%) D	0.41 (±0.01) C	30.7% (±1.5%) AB	8.68 (±0.53) AB	12.57 (±1.01) AB
Mea. fescue	224 (±5.8)	3.6 (±0.1) B	1.59% (±0.06%) D	0.43 (±0.01) C	29.8% (±0.7%) ABC	8.40 (±0.18) B	13.37 (±0.51) AB
Bluegrass	214 (±27.1)	4.4 (±0.5) B	2.07% (±0.02%) C	0.55 (±0.02) B	23.6% (±2.7%) BCD	5.42 (±0.58) C	11.23 (±1.29) BC
Red clover	266 (±13.0)	8.5 (±0.4) A	3.19% (±0.05%) B	0.76 (±0.02) A	21.7% (±2.1%) CD	2.54 (±0.15) D	8.08 (±0.41) CD
White clover	238 (±12.3)	8.4 (±0.4) A	3.52% (±0.07%) A	0.79 (±0.02) A	18.2% (±1.9%) D	2.16 (±0.13) D	7.58 (±0.53) D
L2: Harvested 18 Sep 2017, labeled 18-20 April 2017							
Per. Ryegrass	213 (±4.7) AB	3.0 (±0.1) B	1.42% (±0.02%) D	0.38 (±0.01) C [7]	19.9% (±2.0%) AB	6.58 (±0.58) A	9.30 (±0.79) AB
Timothy	167 (±24.3) B	2.7 (±0.4) B	1.64% (±0.03%) D	0.40 (±0.02) C	18.7% (±2.4%) AB	6.98 (±0.77) A	11.47 (±1.40) A
Tall fescue	268 (±15.2) A	3.8 (±0.2) B	1.41% (±0.07%) D	0.41 (±0.02) C	20.0% (±0.9%) AB	5.34 (±0.18) AB	7.54 (±0.56) BC
Mea. fescue	209 (±14.3) AB	3.4 (±0.3) B	1.64% (±0.02%) D	0.43 (±0.02) C	21.3% (±1.0%) A	6.26 (±0.32) A	10.24 (±0.42) AB
Bluegrass	156 (±11.9) B	3.5 (±0.2) B	2.30% (±0.10%) C	0.55 (±0.01) B	14.4% (±0.8%) BC	4.09 (±0.30) B	9.41 (±0.83) AB
Red clover	269 (±16.6) A	7.8 (±0.6) A	2.88% (±0.09%) B	0.69 (±0.04) A	12.1% (±0.9%) C	1.59 (±0.17) C	4.57 (±0.49) C
White clover	194 (±16.8) B	6.7 (±0.5) A	3.48% (±0.11%) A	0.73 (±0.02) A	9.1% (±0.2%) C	1.37 (±0.10) C	4.77 (±0.35) C

[6] Species effect on DM yield was not significant in L1 H3 ($p=0.16$).

[7] Ryegrass was significantly more N-limited when tested versus meadow fescue in L2 H3 ($p<0.05$).

Table S 7: Pearson Correlation Coefficients and associated p-values between **^{15}N Recovery strength** (percent recovered of label remaining in soil, Eqn. 3) and: **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}) in herbage, **N concentration** in the DM (g N 100 g^{-1} DM), **N status** (2.7. Plant nutritional N status), **RDUI** (^{15}N recovery strength to N yield, Eqn. 4), and **RDM** (^{15}N recovery strength to DM yield, Eqn. 5). Results from both experiments are combined for each harvest period (H1-H6). Groups of all grasses and clovers were analyzed separately.

Pearson Correlation Coefficients Prob > |r| under H0: Rho=0														
	Grasses							Clovers						
Harvest	**N**	**DM yield**	**N yield**	**N conc.**	**N status**	**RDUI**	**RDM**	**N**	**DM yield**	**N yield**	**N conc.**	**N status**	**RDUI**	**RDM**
H1 (L1+L2)	40	0.66138 <.0001	0.57707 <.0001	-0.36167 0.0218	0.31155 0.0504	0.94410 <.0001	0.90697 <.0001	12	0.71917 0.0084	0.70281 0.0108	-0.72323 0.0079	0.59612 0.0408	0.86050 0.0003	0.83134 0.0008
H2 (L1+L2)	40	0.61411 <.0001	0.27962 0.0806	-0.65635 <.0001	-0.22896 0.1553	0.89728 <.0001	0.63917 <.0001	16	-0.09984 0.7130	-0.13000 0.6313	-0.14971 0.5800	-0.13185 0.6264	0.87758 <.0001	0.91843 <.0001
H3 (L1 +L2)	40	0.50563 0.0009	0.20511 0.2042	-0.42519 0.0062	-0.24870 0.1218	0.85333 <.0001	0.78615 <.0001	16	0.54356 0.0295	0.69135 0.0030	0.03261 0.9046	0.52852 0.0353	0.95410 <.0001	0.92677 <.0001
H4 (L1 + L2)	40	0.61933 <.0001	0.59649 <.0001	-0.04791 0.7691	0.45688 0.0030	0.83607 <.0001	0.87164 <.0001	16	0.87370 <.0001	0.87266 <.0001	-0.08301 0.7599	0.63288 0.0085	0.95566 <.0001	0.95663 <.0001
H5 (L1)	20	0.41274 0.0705	0.48141 0.0316	0.00186 0.9938	0.40802 0.0741	0.86027 <.0001	0.80991 <.0001	8	0.65384 0.0786	0.54760 0.1600	-0.64464 0.0844	0.41684 0.3042	0.75048 0.0319	0.73443 0.0380
H6 (L1)	20	-0.30260 0.1947	0.22203 0.3468	0.46162 0.0405	0.42189 0.0639	0.87456 <.0001	0.94360 <.0001	8	0.75138 0.0316	0.70377 0.0514	-0.68094 0.0630	-0.41870 0.3019	0.92347 0.0011	0.70883 0.0490

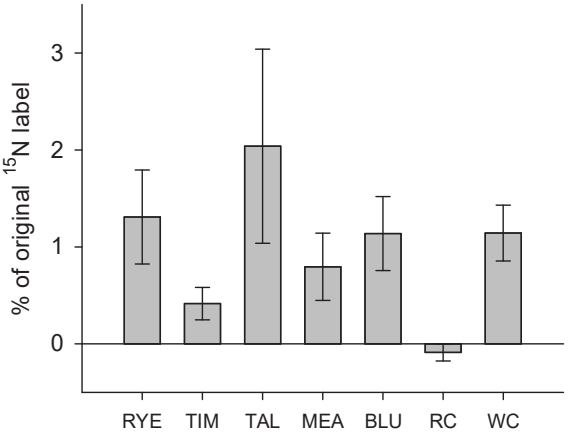


Figure S 1: Percent of original ^{15}N label recovered within 10 cm margin outside the labeled subplot, sum of first three harvests after L1, sum of second and third harvests for white clover which was not harvested in the spring of L1. Mean of 4 replicates (±SE). One-sided t-tests had $p<0.1$ except for red clover, which was not significant. ANOVA showed no significant species effect.

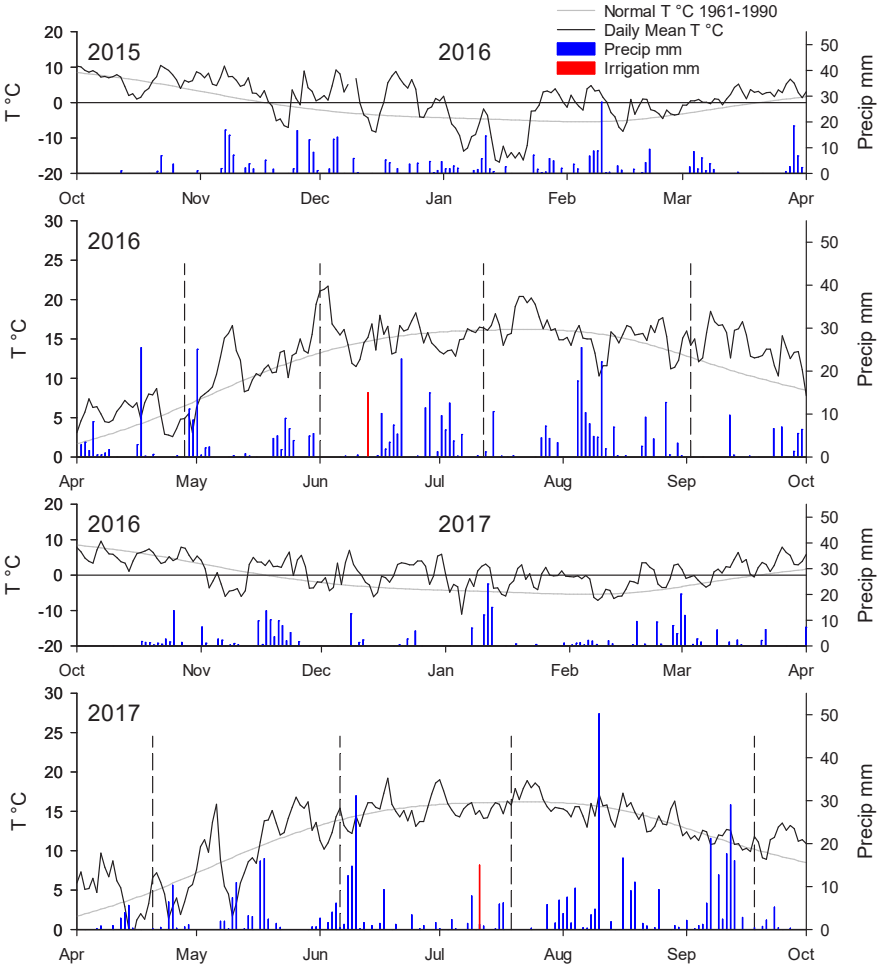


Figure S 2: Weather conditions in preceding winters and throughout growing seasons of L1 (2016) and L2 (2017). Shown: daily mean temperature °C (black lines) and normal mean temperature from 1961-1990 (grey lines). Solid vertical lines show precipitation mm (blue) and irrigation (red). Dashed vertical lines from left to right show date of April ^{15}N labeling and three harvest dates of experimental plots in each year. Weather data was recorded at the nearby NMBU weather station in Ås (59°39'37.8"N, 10°46'54.5"E) (Wolff et al., 2018, 2017, 2016). Drought period: 11-June to 10 July 2017 brought only 32 mm of precipitation.

Text S 1: Explanation of N_{crit} calculation

To evaluate the plant nutritional status taking account for increasing N dilution in growing plants, we estimated N_{crit} for each subplot at each harvest as a decreasing logarithmic function of DM yield m^{-2}, as described by Bélanger and Gastal (2000). We used a function adapted for Norwegian conditions which accounts for the proportion of grasses and clover, and for N partitioning to stubble (ENGNOR model, Baadshaug and Lantinga, 2002). We reprint the equation below, with edited variable names:

$$N_{crit} = (1.0 + \left(\frac{0.23\ CSHARE}{100}\right) \min(5.0, \exp(1.70 - 0.0016(DM_{harveste} + DM_{stubble}))))$$

where

CSHARE: clover percent of DM; only grass = 0, only clover = 100

5.0: maximum N-fraction of young (leaf) tissue, percent of DM

$DM_{harvested}$: weight of harvested herbage, g DM m^{-2}

$DM_{stubble}$: weight of stubble, g DM m^{-2}; we assumed to be 10% of $DM_{harvested}$

Paper II

Deep N acquisition in hemiboreal cultivated grasslands: II. Niche and overyielding effects in mixtures

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina A. Bleken

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Abstract

Aims To explore the contribution of vertical root niche differentiation of different forage species to NH_4^+ recovery below ploughing depth in cultivated grass-clover mixtures.
Methods The recovery of a deep-placed (42 cm) slow-releasing $^{15}NH_4^+$ label was tracked in the herbage of grass-clover mixtures during one growing season and the subsequent spring (3+1 harvests) and compared with performances in pure stands (Byers et al., Manuscript I of this thesis) weighed by the species share in the mixture biomass.
Results Mixtures overyielded in biomass (+11%), total N (+24%), and deep N recovery (+17%). We found evidence of vertical niche differentiation. Tall and meadow fescue, and perennial ryegrass showed positive diversity effects on biomass, N concentration and deep N DM^{-1}. Tall fescue gained the most in deep N DM^{-1}. Other species responded with negative diversity effects on deep N DM^{-1} despite diverse (large or small) deep N recoveries in pure stands. Though deep N DM^{-1} of high-yielding red clover decreased little, due to high yields this translated into a large negative diversity effect on deep N recovery.
Conclusions Grass-clover mixtures best combined yield performance, protein content and recovery of deep-placed $^{15}NH_4^+$. The latter was most pronounced in the autumn and is potentially beneficial for reducing N leaching off-season. Improved N nutrition in grasses due to clover did not prevent positive diversity effect on deep N uptake. Diversity effects were species-specific; those on deep N DM^{-1} and N concentration did not correspond with diversity effects on plant vigor and could not be predicted by performance in pure stands.

1. Introduction

There is an increasing demand for high quality forage grassland yields with high protein content, produced with high nitrogen use efficiency and reduced N losses. Mineral N fertilizer is often transported below the densest root zone, which is roughly 0-20 cm in a typical Norwegian forage grassland (Bleken et al., 2022). Combining species with varied rooting depths is thought to enhance nitrogen utilization throughout the soil profile (Hoekstra et al., 2015; Mamolos et al., 1995; Mommer et al., 2010; Pirhofer-Walzl et al., 2013), while also contributing to the superior performance of mixtures (overyielding) relative to that of components in pure stands (Hoekstra et al., 2015; Mamolos et al., 1995). It has been further theorized that competition between species in the topsoil can stimulate species capable of producing deep roots to increase N uptake from deeper soil (*vertical niche differentiation*, Hoekstra et al., 2015; Mommer et al., 2010). By employing this diversity effect, grassland swards might recover nutrients which have moved below the topsoil by leaching.

Multi-species perennial grassland swards have been reported to realize diversity effects through combinatory species interactions, resulting in enhanced yields or nutrient content, especially when grasses are combined with N-fixing legumes (Hooper, 1998; Jing et al., 2017; Lüscher et al., 2014; Nyfeler et al., 2011). A mixture is said to be overyielding when it yields more than expected from the proportional presence of each component in a mixture (Lüscher et al., 2008; Nyfeler et al., 2011), and transgressively overyielding when yields surpass the highest-yielding species in pure stands (Schmid et al., 2008).

Diversity effects which lead to overyielding are often studied as combinations of functional traits, such as combining differently layered canopies for improving light interception, pairing N-fixing legumes with non-legumes (Nyfeler et al., 2011), mixing quick-establishing with persistent species (Dhamala et al., 2017; Finn et al., 2013), choosing species with growth peaks occurring at different times (temporal niche differentiation, Malcolm et al., 2015; Mamolos and Veresoglou, 2000), and as in this study, combining different rooting depths to best exploit nutrients throughout the soil profile via vertical niche differentiation (Husse et al., 2017; Mommer et al., 2010). We

extend the concept of overyielding in dry matter (DM) yield also to nitrogen (N) yield and to uptake of N from deeper soil.

The phenomenon of vertical niche differentiation is difficult to study and easily confounded with other diversity effects (Hoekstra et al., 2015; Mamolos and Veresoglou, 2000; Pirhofer-Walzl et al., 2013; Ravenek et al., 2014; von Felten et al., 2012; von Felten and Schmid, 2008). Simply increasing the number of species present increases the likelihood that one species will grow well and contribute to overyielding; conversely, certain species may come to dominate a mixture (selection effect), theoretically inhibiting complementarity effects and diminishing overyielding (Cardinale et al., 2007). Positive complementarity effects might however outweigh the effects of species dominance over time, and overyielding has been found to increase with sward age (Fargione et al., 2007). Increased sward age may also heighten risk for negative plant-soil interactions (e.g. disease) in pure stands and low-diversity mixtures (Ravenek et al., 2014). Roots can be studied by measuring root biomass and/or tracing uptake of water or nutrients; both are technically challenging. Presence of roots is not necessarily indicative of active root uptake, and roots can vary in affinity for different molecules (Maire et al., 2009). Furthermore, belowground overyielding of roots may precede aboveground overyielding of herbage (Mommer et al., 2010).

While many studies have confirmed that deep-rooting grassland species can access N from deep soil layers (Hoekstra et al., 2015; Kristensen and Thorup-Kristensen, 2004), mixtures including deep-rooting species do not always obtain more N from deeper soil layers than mixtures without them. Mommer et al. (2010) observed an early increase in root mass but shallower root distributions in mixtures relative to monocultures, which they attributed to inter- and intraspecies root recognition rather than nutritious cues. Hoekstra et al. (2015) found that mixtures including deep- and shallow-rooting species exhibited diversity effects on root uptake, especially under drought, resulting in increased N uptake from 5 cm but not 35 cm depth. Pirhofer-Walzl et al. (2013) observed greater yields and deep ^{15}N recovery by mixtures than by species in pure stands but concluded that legume-sourced N and increasing species diversity in general explained these results better than vertical root complementarity.

Plants normally modify root distribution in order to improve the uptake of limiting nutrients; competition can influence nutrient availability and thus shift the root behavior of species grown in mixtures (Cahill and McNickle, 2011; Hodge, 2004; Robinson et al., 1999). The presence of N-fixing legumes usually increases N concentration in grasses (Nyfeler et al., 2011). This may reduce the competition for N between grasses in a mixture and thus the scope for N uptake from deeper soil layers. On the other hand, Pirhofer-Walzl et al. (2013) observed that when mixed with legumes, perennial ryegrass grew more vigorously and increased ^{15}N uptake from 40 cm depth more than deeper-rooting chicory, suggesting increased competition between the non-legumes for biologically-fixed N. Mamolos et al. (1995) observed that following surface application of mineral N to a nutrient-poor permanent grassland, the most competitive species increased the activity of shallower (5 cm) rather than deeper (15 cm) roots, while less N-competitive species, which they expected to increase in deep root activity, showed no consistent change. Only the legumes present increased deep root activity.

Our study aimed at exploring facilitation, synergy and competition effects on deep N uptake in cultivated grass-clover mixtures grown in a hemiboreal climate typical of southern Norway. To do this, we cultivated pure stands and mixtures including grass species differing in expected rooting depths, N acquisition competitiveness, and persistence under Norwegian conditions, as well as N-fixing clovers. In early spring of the third production year, when the swards were well established, we placed a novel slow-release label consisting of $^{15}NH_4^+$ adsorbed to clinoptilolite (Milovanović et al., 2015) at ~42 cm depth and tracked ^{15}N recovery in herbage harvested over one growing season plus the following spring harvest. The labeling depth was well below the densest root zone; due to the short growing season in Norway, roots of grassland species are expected to grow shallower than in more southern locations. In an adjacent grassland, root density decreased drastically below 15 cm depth, and only 4-6% of the total root biomass from 0-30 cm was found in the compact subsoil below 23 cm (Bleken et al., 2022).

We focus on five well-adapted grass and two clover species. Timothy (*Phleum pratense* L.) and meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.) are the most common ley species in Nordic countries, due to good winter survival and forage quality. Red clover (*Trifolium pratense* L.) is the dominating leguminous ley species in Norway and

reputed to be deep rooting due to its persistent taproot (Ergon et al., 2016). We included perennial ryegrass (*Lolium perenne* L.) because of its high yield potential and very good nutritional value, though it is less common in Norway due to poor winter survival (Østrem et al., 2015). Ryegrass is often described as shallow-rooting (Pirhofer-Walzl et al., 2013). Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort) on the other hand is reputed to be deep rooting and drought resistant (Hernandez and Picon-Cochard, 2016; Malcolm et al., 2015). For further contrast we included shallow-rooting Kentucky bluegrass (*Poa pratensis* L.) and white clover (*Trifolium repens* L. cv.), commonly included in pastures, where they grow low in height and spread by creeping expansion of rhizomes and stolons, respectively.

In a companion article (Byers et al., Manuscript I of this thesis) we evaluate our slow-release $^{15}NH_4^+$ labeling method and present results for species in pure stands including their response to weather events. In the present article we investigate the potential for overyielding in two grass-clover mixtures and focus on diversity and vertical niche effects realized by the mixture components, using results in pure stands from the same year as a baseline. We also consider aboveground DM and N overyielding due both to its agronomical relevance and the interdependence of N uptake, photosynthesis, and root growth.

The following hypotheses were tested: (1) Mixtures of grasses and clover will overyield in aboveground herbage and N per area; (2) Mixtures will recover more deep-placed ^{15}N than pure stands; (3) When exposed to competition in mixtures also containing clovers, grasses will increase in N concentration, but nonetheless exhibit vertical niche differentiation as follows: purportedly deep-rooting species (tall fescue, meadow fescue, and timothy) will increase uptake of deep-placed ^{15}N, while perennial ryegrass and bluegrass will decrease uptake of deep-placed ^{15}N in mixtures compared to in pure stands; (4) Alternatively, increased N concentration in grasses due to presence of clovers in the mixture may suppress vertical niche differentiation in N uptake by the grasses; (5) The clovers will decrease uptake of deep-placed ^{15}N in mixtures compared to in pure stands.

2. Materials and methods

2.1 Site and sward management

The experiment was established in a stone-free Umbric Epistagnic Retisol (IUSS, 2015) at the NMBU research farm in Ås, Norway (59°39'49"N, 10°45'38"E, 69 m a.s.l.), which is artificially drained at 1 m depth. The bulk density is low in the top layer (1.15 g cm^{-2}), but increases markedly below the ploughing depth (20 cm) to 1.5 g cm^{-2} at 40 cm. During the growing season from May through September, the mean temperature normal (1991-2020) is 13.8 °C, while it was 13.7 °C in 2017. The mean temperature normal for May is 10.7°C, while in May 2018 it was a record-high 15.3°C. For October through April, the mean temperature normal is 0.9°C, while it was 1.5°C and 0.2°C during the winters leading to spring 2017 and 2018; the winter leading to 2018 was more harsh, with continuous snow cover from early January to early April (Byers et al., 2021). Plants were exposed to drought in April and July 2017. There was an exceptionally heavy rain event of ~55 mm in August 2017 between the second and third harvests. Weather data are from the nearby weather station Ås (No. SN17850) at 59°39'37.8" N, 10°46'54.5" E, 94 m a.s.l. (Wolff et al., 2021, 2018, 2017).

Pure stands and mixtures of forage grass and clover species were sown in June 2014 in fully randomized plots (8 m x 1.5 m, 12 sowing rows per plot). Clover seed was inoculated using soil from an organically managed crop rotation. Starting in 2015, the swards were harvested three times per year following local recommendations for high forage digestibility. All treatments received a moderate N fertilizer dose, in total 200 kg N ha^{-1} yr^{-1} applied as NPK 22-3-10, distributed 40% in early spring before H1 (60% in 2018), 40% after H1 and 20% after H2 (Table 1).

More details about the field management, soil and weather are reported in Byers et al. (Manuscript I of this thesis).

Table 1: Dates of ^{15}N label application, herbage harvest and N fertilization. For each harvest, cumulated rainfall + irrigation in mm and growing degree days, basis 5°C from 1 April, are given for the period spanning from the previous harvest or 1 April in the case of the spring harvests.

Operation	**2017** (3rd prod. year)	**2018** (4th prod. year)
Spring fertilization [a]	2 May 80 kg N ha^{-1}	27 April 120 kg N ha^{-1}
^{15}N application	18-20 April	
H1, H4: Spring harvests	6 June (125 mm, 499 GDD)	30 May (65+30 mm, 556 GDD)
80 kg N ha^{-1}	19 June	
H2: Summer harvest	19 July (107+15 mm, 640 GDD) [b]	
40 kg N ha^{-1}	27 July	
H3: Autumn harvest	18 Sept. (260 mm, 884 GDD)	

[a] Spring fertilization was increased to 120 kg N ha^{-1} in 2018.
[b] During a drought period from 11 June to 10 July 2017 there was only 32 mm of precipitation and no irrigation until just before H2.

2.2 Treatments and ^{15}N Labeling

This article reports the results from two ley mixtures and compares performance of seven of the component species to their performance in pure stands, which are presented in the companion article (Byers et al., Manuscript I of this thesis). Component species were sown in equal seed proportions by weight. **Mix 4** was composed of timothy (*Phleum pratense* L. cv. Grindstad), perennial ryegrass (*Lolium perenne* L. cv. Figgjo), tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort. cv. Swaj), and red clover (*Trifolium pratense* L. cv. Lea). **Mix 10** in addition to the four species of Mix 4 included meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv. cv. Fure), Kentucky bluegrass (*Poa pratensis* L. cv. Knut), white clover (*Trifolium repens* L. cv. Milkanova), smooth bromegrass (*Bromus inermis* Leyss.), festulolium (Hybrid *Festuca x Lolium*), and alfalfa (*Medicago sativa* L.). No companion ^{15}N-labeling study was carried out on pure stands of alfalfa, festulolium, or bromegrass.

^{15}N labeling of each treatment was replicated in 4 subplots on 4 fully-randomized sowing plots. A slow-release label of 98 atom percent (AT%) $^{15}NH_4^-$ adsorbed to clinoptilolite was applied at 42 cm depth in early spring (18-20 April 2017) of the third production year, before the onset of the spring regrowth; see Byers et al. (Manuscript I of this thesis) for details. Each subplot received a dose of 36 mg ^{15}N, which translates to 68 mg m^{-2} of plant sampling area. To label subplots, we pre-augured a 4 x 4 array of 16 mm diameter, 0.43 m deep holes centered between sowing rows and spaced 12 cm apart. Care was taken to avoid contamination of the soil above the label and to tightly fill the holes with finely ground and compacted dry clayey soil from the same field, which expands on rewetting, to hinder preferential root growth through the hole.

2.3 Plant sampling and N, ^{15}N recovery

We measured the recovery of ^{15}N in the herbage in the three harvests of the growing season following the labeling event (H1, spring; H2, summer; and H3, autumn), and in the spring harvest of the following growing season (H4). The herbage was separated into individual species while fresh, dried at 40-60°C, and chopped; a subsample was ground, ball-milled and analyzed for N content and excess ^{15}N above natural abundance. Details about the analyses and equations for estimating N and label ^{15}N uptake in the herbage are given in Byers et al. (Manuscript I of this thesis).

Per mixture subplot *j*, the total DM yield (g m^{-2}), N yield (g m^{-2}) and ^{15}N herbage uptake (mg m^{-2}) are the summed values of the component species *i*:

$$(Yield\ or\ ^{15}N\ herbage\ uptake)_j = \sum_i (Yield\ or\ ^{15}N\ herbage\ uptake)_{ij} \quad (1)$$

The yield-weighted annual ^{15}N herbage uptake per DM of each subplot *j* over multiple harvests was calculated for each species *i* at each harvest *h*:

$$(Yield\text{-}weighted\ mg\ ^{15}N\ g^{-1}\ DM)_j = \frac{\sum_{h,i}\ (mg\ ^{15}N\ m^{-2})_{hij}}{\sum_{h,i}\ (g\ DM\ m^{-2})_{hij}} \quad (2)$$

The same approach was used for annual ^{15}N herbage uptake per N (mg ^{15}N g^{-1} N), and annual N concentration in DM (g N g^{-1} DM).

2.4 Species diversity effects

In this study we call *diversity effect* the difference between the *realized* performance of a species grown in a mixture and that which is *expected* based on the species performance in pure stands. For each species *i* and subplot *j, diversity effects* were calculated as:

$$(Diversity\ effect)_{ij} = (Realized\ value\ in\ mixture)_{ij} - (Expected\ value)_{ij} \quad (3)$$

where *value* can be DM yield (g DM m^{-2}), N yield (g N m^{-2}), or the ^{15}N uptake in herbage (mg ^{15}N m^{-2}). The expected DM and N yields m^{-2} are the yields realized in pure stands weighted by the proportion of DM the species occupies in the mixture:

$$(Expected\ g\ DM\ or\ N\ yield\ m^{-2})_{ij} = (DM\ share\ in\ mixture)_{ij} * (Mean\ pure\ stand\ value)_i \quad (4)$$

In the case of ^{15}N uptake in herbage, the expected value was both weighted by proportion of DM and the amount of ^{15}N remaining in the soil after each harvest, which could differ between mixtures and pure stands:

$$\begin{aligned}(Expected\ mg\ ^{15}N\ uptake\ m^{-2}\ by\ mixture\ component)_{ij} &= (DM\ share\ in\ mixture)_{ij} \\ &* Mean\left(\frac{mg\ ^{15}N\ uptake\ m^{-2}}{mg\ ^{15}N\ m^{-2}\ remaining}\right)_{pure\ stands,\ i} \\ &* \left(mg\ ^{15}N\ m^{-2}\ remaining\ in\ mixture\ subplot\right)_j\end{aligned} \quad (5)$$

The diversity effect on total N or ^{15}N uptake in mixtures can result from changes in DM yield, N concentration, or changed deep root foraging behavior relative to pure stands. A positive diversity effect on N concentration or ^{15}N concentration in DM does not necessarily indicate a competitive advantage in total or deep N acquisition, as it could co-occur with reduced plant growth (negative diversity effect on DM). To explore this we estimated the ^{15}N Recovery per Dry Matter (RDM) as:

$$RDM_{ij} = \left(\frac{mg\ ^{15}N\ uptake\ m^{-2} / mg\ ^{15}N\ m^{-2}\ remaining}{DM\ yield\ \mathrm{m}^{-2}} \right)_{ij} \quad (6)$$

Similarly, to explore how the plants' utilization of N_{min} from deeper soil contributes to their total N acquisition, we estimated the Relative Deep Uptake Index (RDUI) as:

$$RDUI_{ij} = \left(\frac{mg\ ^{15}N\ uptake\ m^{-2} / mg\ ^{15}N\ m^{-2}\ remaining}{N\ yield\ \mathrm{m}^{-2}} \right)_{ij} \quad (7)$$

RDM and RDUI account for the ^{15}N remaining in each subplot after previous harvests, and are weighted by yield, and can thus be compared directly between mixture components and pure stands. The diversity effects on RDM and RDUI were calculated as:

$$(Diversity\ RDM\ or\ RDUI)_{ij} = (Value\ in\ mixture)_{ij} - (Mean\ pure\ stand\ value)_i \quad (8)$$

Notice that increased N concentration reduces or makes negative the diversity effect on RDUI, without necessarily indicating reduced deep root uptake of N.

2.5 Mixture overyielding

The mixtures' overyielding in DM yields, N yields and ^{15}N uptake m^{-2} was defined as the difference between the yield of the mixture subplot and the sum of the expected values of all species *i* present in the mixture (which is equal to the sum of the diversity effects):

$$Overyielding_j = Mixture\ yield_j - \sum_i (Expected\ value)_i \quad (9)$$

Transgressive overyielding occurs when a mixture surpasses the performance of the best pure stands.

This definition of overyielding differs from Ergon et al. (2016), Kirwan et al. (2009), and Nyfeler et al. (2011), who used *expected* yields proportional to the seed proportions at sowing. The use of *realized* proportions of DM herbage yield in the mixtures

accommodates for the marked shift in botanical composition from the original seeding proportions by the third production year.

No ^{15}N-labeling experiment was carried out in pure stands of bromegrass (Byers et al., Manuscript I of this thesis) although it was found in small amounts in some Mix 10 subplots (See Methods: Treatments and ^{15}N labeling). We included bromegrass in overyielding calculations assuming it had no diversity effects, i.e. its *expected* values were assumed equal to its *realized* values. Weeds were excluded.

2.6 Soil mineral N content

Soil was sampled when preparing the holes for the ^{15}N label placement (at 0-20, 20-30 and 30-43 cm depths), and sampled again in the autumn (30 October 2017) with a hydraulic press from unlabeled areas of the main plots hosting the subplots (at 0-20, 20-40 and 40-60 cm depths, four soil cores per plot). The soil was immediately refrigerated to 4°C, sieved to 2.0 mm, and analyzed for 1 M KCl-extractable NH_4, NO_2 and NO_3-N using colorimetric assays on a Tecan Infinite F50 microplate photometer (Byers et al., Manuscript I of this thesis).

2.7 Statistics

Analyses of variance were performed using the R software package, version 3.6.1. We used *post hoc* Tukey tests to compare cumulative annual results of mixtures and pure stands (using simple.glht from the mixlm package for R, α=0.05). Whole-mixture positive overyielding of DM, N and ^{15}N uptake was assessed by one-sided t-tests (α=0.05), separately for Mix 4 and Mix 10, and pooled together. Diversity effects on yields of individual species (which when summed, equal whole-mixture overyielding) were assessed separately per mixture by two-sided t-tests. Diversity effects on N DM^{-1}, ^{15}N DM^{-1} and ^{15}N N^{-1} were assessed both separately and pooled together from both mixtures by two-sided t-tests. Statistically significant effects ($p \leq 0.05$) are for brevity referred to as 'significant' effects.

3. Results

3.1 Mixture composition

The contribution of the single components to the total mixture DM yield diverged strongly from the original seeding proportion and changed from harvest to harvest (Fig. 1). Mix 4 was dominated by red clover and perennial ryegrass, with timothy and tall fescue comprising around 16% in the first year's three harvests (H1 to H3) and increasing their share along with ryegrass in the spring harvest of the second year (H4) after prolonged early spring snow cover had strongly diminished red clover. Mix 10 was also dominated by red clover, while timothy, ryegrass and both fescues (hereafter called the tall-growing grasses) were more evenly distributed.

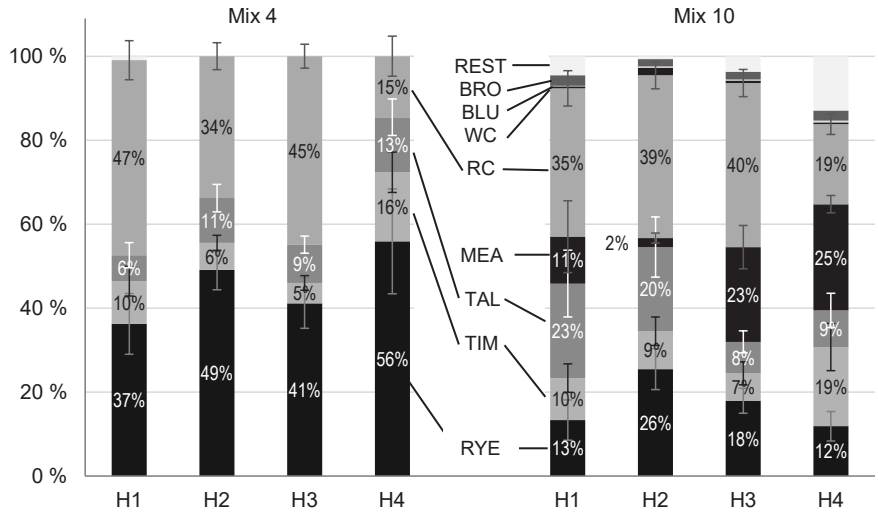


Figure 1: Component species' share of DM yield in Mix 4 and Mix 10, by harvest. Average share in % of total DM (±SE except for species present only in small amounts: very little bluegrass, bromegrass, or white clover was found in Mix 10). Festulolium was not successfully identified during sorting and might be included in the fescues. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover, REST: weeds. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018.

The harsh winter leading up to H4 also diminished red clover in Mix 10, which became dominated by timothy and the fescues along with an increased weed presence, though

ryegrass did not increase as it did in Mix 4. Ryegrass doubled its DM contribution to both mixtures from H1 to the drought-affected summer harvest H2; red clover also yielded strongly in H2 (Suppl. Table S 2) and only showed a reduced proportion in Mix 4 due to ryegrass dominating. Timothy contributed about the same DM share per harvest in both mixtures.

Only small amounts of bluegrass, white clover and bromegrass survived early competition and were found in Mix 10 in at least two replicate plots at each harvest. Alfalfa established poorly and disappeared due to failed rhizobial inoculation. Festulolium was not identified during sorting and if present was likely included among the fescues.

3.2 Overyielding of mixtures

Mixtures overyielded in annual DM, N, and uptake of ^{15}N in herbage (on average +11%, +24% and and 17%, respectively). Though not surpassing the highest-yielding pure stands in a single measure, mixture overyielding was balanced across DM, N and ^{15}N such that their overall performance was stronger than that of pure grasses or pure clovers. However, Mix 4 transgressively overyielded in ^{15}N uptake in the autumn, as discussed below. Mixtures showed the largest annual (H1 to H3) DM yields (>900 g DM m^{-2}), even though the advantage over the highest DM-yielding pure stands red clover, tall fescue and timothy was not significant (Table 2).

The annual N concentration (weighted ratio, Eqn. 2) of the mixtures was on average ~2.6% of DM, closer to that of clover pure stands than tall-growing grasses, and consequently the mixtures' N yields (~24 g N m^{-2}) were similar to the N yields of the clovers (Table 2). Mix 4 recovered 52% of the applied ^{15}N, not significantly different from the best-recovering pure stands (timothy; 55%), while Mix 10 recovered 43%, similar to tall and meadow fescue in pure stands.

Table 2: Cumulative annual DM yield (g DM m^{-2}), **N yield** (g N m^{-2}), and total **Herbage uptake ^{15}N** (mg ^{15}N m^{-2}) of three consecutive harvests after ^{15}N labeling in early spring 2017, and related yield-weighted ratios (Eqn. 2): **Herbage uptake ^{15}N to N yield** (mg ^{15}N g^{-1} N), **Herbage uptake ^{15}N to DM yield** (mg ^{15}N g^{-1} DM), and **N concentration in the DM** (g N 100 g^{-1} DM). Mean values of 4 replicates (±SE), weeds excluded. Letters indicate *post hoc* Tukey groupings (α=0.05).

Cumulative total 3 harvests (±SE) Tukey α=0.05	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	Herbage uptake ^{15}N (mg ^{15}N m^{-2})	Herbage uptake ^{15}N/N yield (mg ^{15}N g^{-1} N)	Herbage uptake ^{15}N/DM yield (mg ^{15}N g^{-1} DM)	N conc. (g N 100 g^{-1} DM)
Mix 4	**933 (±9.4) A**	**24.7 (±0.6) A**	**35.3 (±0.8) AB**	**1.43 (±0.02) CD**	**0.038 (±0.001) ABC**	**2.64 (±0.07) B**
Expected	*836 (±9.9)*	*19.3 (±0.4)*	*27.8 (±0.6)*			
Mix 10	**908 (±60.7) AB**	**23.8 (±2.1) A**	**29.2 (±0.3) B**	**1.25 (±0.10) DE**	**0.033 (±0.002) CDE**	**2.61 (±0.07) BC**
Expected	*842 (±13.4)*	*19.5 (±0.6)*	*27.8 (±0.5)*			
Pure stands						
Per. Ryegrass	749 (±28.1) BCD	12.3 (±0.4) B	33.5 (±0.9) AB	2.73 (±0.11) A	0.045 (±0.002) A	1.64 (±0.02) D
Timothy	851 (±17.8) ABC	15.7 (±0.6) B	37.6 (±1.5) A	2.42 (±0.14) AB	0.044 (±0.002) AB	1.84 (±0.05) D
Tall fescue	880 (±27.9) ABC	15.6 (±1.0) B	29.2 (±0.9) B	1.90 (±0.17) BC	0.033 (±0.002) BCD	1.78 (±0.12) D
Meadow fescue	742 (±24.6) CD	14.2 (±0.7) B	31.0 (±2.6) AB	2.21 (±0.23) AB	0.042 (±0.004) ABC	1.91 (±0.04) D
Bluegrass	645 (±8.5) D	14.9 (±0.4) B	21.0 (±2.2) C	1.41 (±0.14) CD	0.032 (±0.003) CDE	2.31 (±0.06) C
Red clover	892 (±49.8) ABC	27.8 (±2.2) A	19.5 (±0.5) C	0.71 (±0.05) E	0.022 (±0.001) E	3.10 (±0.07) A
White clover	686 (±39.2) D	22.1 (±1.3) A	16.3 (±0.6) C	0.75 (±0.07) E	0.024 (±0.002) DE	3.22 (±0.03) A

The weighted annual ^{15}N per total N in both mixtures was significantly smaller than in the pure stands with greatest $^{15}N\ N^{-1}$ (ryegrass, timothy and meadow fescue), similar to that of tall fescue and bluegrass and about double that of the clovers (Table 2). By herbage dry matter, Mix 4's weighted annual $^{15}N\ DM^{-1}$ was only slightly less (not significantly different) than that of the pure stands with greatest $^{15}N\ DM^{-1}$ (ryegrass, timothy and meadow fescue), while Mix 10 had $^{15}N\ DM^{-1}$ similar to that of tall fescue and bluegrass, and about 50% greater than that of the clovers (Table 2).

The annual N concentration (weighted ratio, Eqn. 2) of the mixtures was on average ~2.6% of DM, closer to that of clover pure stands than tall-growing grasses, and consequently the mixtures' N yields (~24 g N m^{-2}) were similar to the N yields of the clovers (Table 2). Mix 4 recovered 52% of the applied ^{15}N, not significantly different from the best-recovering pure stands (timothy; 55%), while Mix 10 recovered 43%, similar to tall and meadow fescue in pure stands. The weighted annual ^{15}N per total N in both mixtures was significantly smaller than in the pure stands with greatest $^{15}N\ N^{-1}$ (ryegrass, timothy and meadow fescue), similar to that of tall fescue and bluegrass and about double that of the clovers (Table 2). By herbage dry matter, Mix 4's weighted annual $^{15}N\ DM^{-1}$ was only slightly less (not significantly different) than that of the pure stands with greatest $^{15}N\ DM^{-1}$ (ryegrass, timothy and meadow fescue), while Mix 10 had $^{15}N\ DM^{-1}$ similar to that of tall fescue and bluegrass, and about 50% greater than that of the clovers (Table 2).

Mineral N in the soil of mixture subplots at the time of ^{15}N labeling in April 2017 was similar to that of grass pure stands, and about half that of the clover subplots. By the end of the season in October, soil mineral N was slightly higher in Mix 4 subplots than in those of ryegrass or tall fescue, but still less than half that in red clover (Suppl. Table S 1, only four treatments sampled in October). More details about redistribution of ^{15}N label and mineral N in the soil profile are given in Byers et al. (Manuscript I of this thesis).

Compared to expected values (Eqn. 3-5), annual yields of the two mixtures together overyielded significantly (Eqn. 9), with +10% DM yield, +25% N yield, and +16% ^{15}N recovery (Table 3 A). Including H4 gave similar results (Table 3 B). However, Mix 10 exhibited weaker overyielding than Mix 4, particularly with respect to deep ^{15}N recovery, which, while significant, was only 5% more than expected, compared to 27%

more in Mix 4 annually (Table 3 A). The lack of significance of the ~8% DM overyielding in Mix 10 can be explained by its large yield variability related to patchiness of its herbage cover (SE ±60 g m^{-2}, Table 2).

Table 3: Cumulative overyielding from 3 or 4 harvests as percent increase from expected values of **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}), total **^{15}N herbage uptake** (mg ^{15}N m^{-2}) of three (A) and four (B) harvests after ^{15}N labeling. Mean percent increase of 4 replicates relative to expected values, (±SE). Note that probability value (p) of one-sided t-tests for positive overyielding were calculated on absolute differences rather than percentages. Also: **Percent of ^{15}N label recovered** in harvested herbage in total, and **due to overyielding**. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018.

	Overyielding (±SE) as % increase from expected value			Recovered ^{15}N	
Mixture	DM yield	N yield	^{15}N Recovery	% of label applied	Due to overyielding
A) First year, H1 to H3					
Both	**+9.7% (±2.8%), p=0.006**	**+24.8% (±4.0%), p<0.001**	**+16.1% (±4.7%), p=0.006**	**47.4**	**6.6**
Mix 4	+11.6% (±1.2%), p=0.001	+27.8% (±3.1%), p=0.001	+27.0% (±4.7%), p=0.005	51.9	11.0
Mix 10	+7.8% (±5.8%), p=0.135	+21.8% (±7.6%), p=0.032	+5.1% (±1.5%), p=0.020	42.9	2.1
B) All harvests, H1 to H4					
Both	**+11.1% (±4.3%), p=0.018**	**+24.1% (±4.9%), p=0.001**	**+16.8% (±3.8%), p=0.002**	**51.7**	**7.4**
Mix 4	+12.6% (±2.7%), p=0.009	+25.5% (±2.1%), p=0.001	+25.9% (±4.0%), p=0.004	54.9	11.3
Mix 10	+9.7% (±8.8%), p=0.175	+22.7% (±10.2%), p=0.056	+8.0% (±1.8%), p=0.010	48.5	3.6

The mixtures' DM and N yields were relatively evenly distributed over the four harvests taken (H1-H4, Suppl. Fig. S 1). DM overyielding was moderate in the springs (significant only in Mix 10, H1), absent or negative in the drought-affected summer harvest and large in autumn (~28% above expected, Suppl. Fig. S 1). The mixtures generally overyielded N at every harvest, except for Mix 10 in the summer harvest (H2). N overyielding was strongest in autumn (48% more than expected in Mix 4, 41% in Mix 10). The recovery of ^{15}N by mixtures varied widely between harvests; it was weakest in

the springs (~6% of applied ^{15}N in H1, 6-10% in H4) and varied between ~20-30% of remaining ^{15}N in H2 and H3 (Suppl. Fig. S 1). Overyielding of ^{15}N was very large in the autumn: 62% more than expected by Mix 4, 47% more by Mix 10, which was more than the total N overyielding in the same harvest, which in turn exceeded DM overyielding. By contrast, in the spring harvests there was no significant ^{15}N overyielding, and in the summer harvest only Mix 4 overyielded while Mix 10 underyielded ^{15}N uptake, as seen for DM yield (Suppl. Fig. S 1).

3.3 Species diversity effects contributing to overyielding

The diversity effects (Eqn. 3-4) for a species express the extent of influences on that species by being grown in a mixture, and thus the species' contribution to mixture overyielding (Eqn. 9). The diversity effects on DM and N yields were mostly positive or neutral, however diversity effects on DM yields were small (Mix 4) or negative (Mix 10) in the summer harvest (Fig. 2, p-values available in Suppl. Table S 2). Ryegrass followed by red clover showed the strongest positive diversity effects on DM, and ryegrass showed even stronger positive effect on N yields. In the autumn harvest of Mix 10, the fescues also showed strong effects contributing to total DM and N overyielding. Timothy, which in pure stands had the best spring yield, responded negatively in mixtures in the spring harvests and contributed little to the DM overyielding in summer and autumn. Timothy also showed mainly negative or no diversity effects on N yield. Bluegrass and white clover were found in small amounts in Mix 10 and no substantial diversity effects on DM or N yield could be discerned.

The diversity effects on ^{15}N uptake in herbage (Eqn. 3, 5) discriminated species with positive response, ryegrass, tall and meadow fescue, from red clover and timothy, which showed clear overall negative responses (Fig. 2, Suppl. Table S 2). The negative diversity effect on deep ^{15}N recovery differentiated timothy from the other tall-growing grasses. Ryegrass dominated the positive diversity effects on ^{15}N recovery in Mix 4, whereas it contributed little in Mix 10, where the fescues together dominated in positive diversity effects. White clover and bluegrass were scarcely present in Mix 10 and therefore expressed no or small negative diversity effects on ^{15}N uptake.

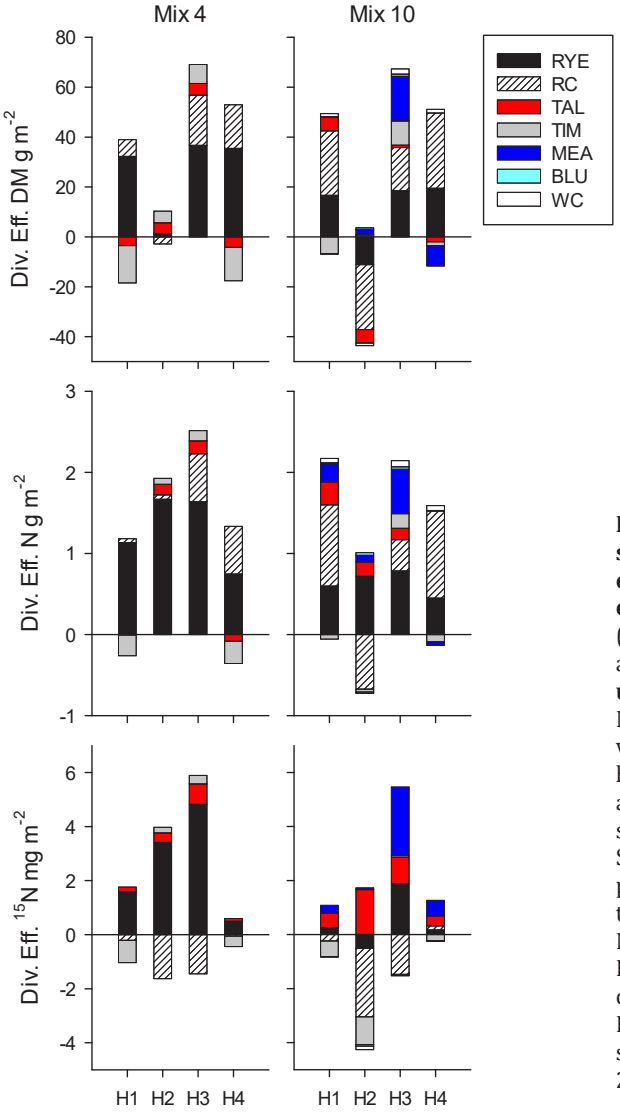


Figure 2: Component species' diversity effects contributing to overyielding of DM yield (g m^{-2}), **N yield** (g N m^{-2}) and total **^{15}N herbage uptake** (mg ^{15}N m^{-2}) in Mix 4 and Mix 10. Mean values of 4 replicates by harvest. Standard errors and significance of two-sided t-tests are shown in Suppl. Table S 2. RYE: perennial ryegrass; TIM: timothy; TAL: tall fescue; MEA: meadow fescue; BLU: bluegrass; RC: red clover; WC: white clover. H1: spring 2017; H2: summer 2017; H3: autumn 2017, H4: spring 2018.

3.4 Diversity effects on concentrations of nitrogen and deep N uptake (RDM and RDUI)

Diversity effects on N concentration in the grass herbage were positive, except for timothy in some harvests. The increase in N concentration was greatest in bluegrass (short growing) and ryegrass (tall growing). Overall, clovers responded little, with some small positive or negative effects (Suppl. Table S 2).

Most species, pooled together from both mixtures and tested separately per harvest (H1-H4) showed significant effects, positive or negative, on ^{15}N Recovery per DM (RDM, Eqn. 6, 8), or at least strong tendencies to significant effects ($0.05<p\leq0.10$; Suppl. Table S 2). Red and white clover, timothy and bluegrass consistently reduced RDM, while tall fescue and ryegrass increased it, except in the first spring harvest (H1) when ryegrass showed no response. Meadow fescue showed no response in the two first harvests and increased its RDM in the autumn harvest (H3) and in the spring harvest of the second year (H4). Diversity effects on RDM were large in summer and largest in autumn, and smaller but often significant in the spring harvests H1 and H4 (Suppl. Table S 2). Overall, ryegrass and the fescues increased annual weighted RDM (Eqn. 2) when cultivated in mixtures (Fig. 3), while the other species responded negatively, including the tall-growing timothy, which in pure stands was among the highest ^{15}N-uptaking species (Byers et al., Manuscript I of this thesis).

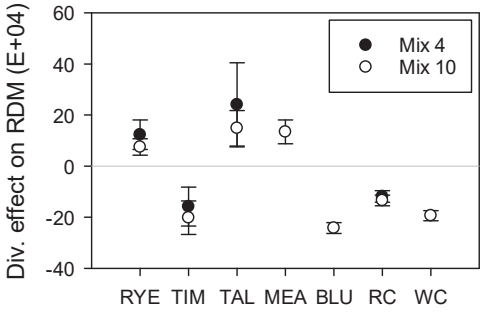


Figure 3: Cumulative diversity effects on RDM (*realized - expected* values): ^{15}N recovery per Dry Matter (Eqn. 6, 8; mg ^{15}N g^{-1} DM), shown in each species in Mix 4 and Mix 10. Weighted average (Eqn. 2) of four replicates for all four harvests taken, ±SE.

To explore other factors contributing to deep N uptake, we looked at the relationships between species diversity effects on ^{15}N recovery, whether as mg m^{-2} (Eqn. 3, 5) or

RDM, and diversity effects on DM and N yield, N concentration and RDUI (Eqn. 7, 8), which is ^{15}N uptake weighted by N yield rather than DM yield. Plotting all observations at all four harvests taken, the species showed distinct patterns of diversity responses (Fig. 4, shown in 4.2 Factors contributing to overyielding), ranging from nil to strong positive or negative values for RDM, while RDUI increased only in tall fescue and decreased in all other species when results from both mixtures were pooled together (Suppl. Table S 2). The response patterns will be analyzed in the Discussion.

4. Discussion

4.1 Overyielding in mixtures

In total, the grass-clover mixtures surpassed the weighted average of their components with respect to biomass and nitrogen yield as well as deep N uptake, confirming the overyielding hypotheses (Table 3). Although the mixtures did not achieve transgressive annual overyielding in any of the single traits considered (DM, N or ^{15}N per land area), in most cases their performance was similar to that of the pure stands with highest absolute value (Table 2). Thus, the mixtures were superior to the pure stands as they showed the best combination of DM and N yields and ^{15}N uptake in the same sward. Mixtures were also superior to pure stands (Suppl. Table S 2 and Byers et al., Manuscript I of this thesis) as they provided a more even DM yield and protein concentration over the three harvests, i.e. gave lower intra-annual variation in DM yield and quality, in agreement with findings by Ergon et al. (2016) and Hooper (1998).

The three overyielding traits considered are intrinsically interconnected, in addition to being focal for the agronomic outcome and N losses. As all grasses exhibited some degree of N deficiency in pure stands (Byers et al., Manuscript I of this thesis), the increased N concentration observed in grasses when grown with clovers is in itself a cause of increased productivity, including root growth. Increased N concentration also improves the forage quality since protein yield is directly proportional to N content.

The overyielding of ^{15}N recovery indicates a heightened ability of mixtures to recover nutrients and in particular mineral N leached below the ploughed layer. We expect that the positive complementarity effect observed with $^{15}NH_4^+$ would be present also in the case of the more mobile NO_3^-. Noteworthy, in the autumn, when grass species in pure

stands showed the most severe N deficiency (Byers et al., Manuscript I of this thesis), ^{15}N overyielding was transgressive and surpassed N overyielding (Suppl. Fig. S 1). Soil analysis in the early spring and at the end of the growing season in autumn confirmed that mixtures exploited soil mineral N as well as the grasses and better than the clovers (Suppl. Table S 1). Thus the grasses in the mixtures alleviated the exacerbating effect clovers have on N leaching and its impact on eutrophication, and the greenhouse gas nitrous oxide, N_2O. This is important because under hemiboreal conditions, freeze-thaw enhances N_2O formation also below ploughing depth (Bleken et al., 2022; Byers et al., 2021), and N_2O emissions during winter have been found to contribute the a majority of total annual emissions (Reinsch et al., 2018).

Overyielding of deep N uptake in the spring harvests H1 and H4 were small, mirroring our finding (Byers et al., Manuscript I of this thesis) that regrowth from new tillers in the spring delays the formation of secondary roots (Chen et al., 2016) and deep root activity, and that increasing depth of active root uptake throughout the season may also occur in clovers, including tap-rooting red clover, in agreement with observations by Husse et al. (2017). Mommer et al. (2010) attributed an early increase in root mass but shallower root distributions in mixtures to inter- and intraspecies root recognition rather than nutritious cues.

4.2 Factors contributing to overyielding

4.2.1 Complementarity effects versus species dominance

Several complementarity effects might have contributed to overyielding (Hooper, 1998), for example increased light interception and use efficiency due to better foliar architecture. Low height and thus weak competition for light can explain the poor performance of bluegrass in mixtures with tall-growing grasses and red clover. Because of its low but dense herbage, bluegrass is more adapted to grazing conditions than to competition with tall species. Temporal complementarity (Husse et al., 2017, 2016) is supported by our observation that the species which recovered the most ^{15}N in pure stands (Byers et al., Manuscript I of this thesis), and diversity effects on ^{15}N recovery in mixtures (Suppl. Table S 2), varied from harvest to harvest. A complete analysis is beyond the scope of this study, which focused on diversity effects on the N yield and deep N uptake.

Any diversity effect facilitating a species' growth in a mixture will increase its competitiveness, potentially contributing to overyielding but also to the depression of other species (Cardinale et al., 2007). We observed that the non-winter hardy ryegrass overwintered better in mixtures, and in early spring the clovers regenerated their canopy more rapidly in mixtures. The latter could be partly explained by better soil moisture at the start of spring regrowth (data not shown) due to more plant mulch which mitigated the effect of strong solar radiation and wind on evaporation. Furthermore, foliar diseases which affected grasses in the autumn harvest showed a marked decrease in mixtures compared to pure stands (data not shown). Mixtures overyielded despite evidence of species dominance by ryegrass and red clover in Mix 4, and by red clover and tall-growing grasses in Mix 10 (Fig. 1), in accordance with the theory that diversity effects outweigh species dominance over time (Fargione et al., 2007). The fact that Mix 4 performed better than Mix 10 is in agreement with previous observations that although productivity generally increases with species diversity, species composition may be more important, and the expression of positive functional interaction depends on the environmental conditions (Hooper et al., 2005).

It was surprising that while winter-hardy timothy had high DM yields, N yields and ^{15}N uptake in pure stands, in mixtures it was initially outcompeted by aggressive growth of ryegrass which was more competitive for N (Fig. 4 B). Maire et al. (2009) observed that timothy is less competitive for NH_4^+ than perennial ryegrass but more competitive for NO_3^- and NH_4^+ than tall fescue. Timothy is the most commonly sown grass species in Fennoscandia, often together with meadow fescue, and is also commonly used in Canada due to its winter hardiness. Contrary to common local practice our experiment was established in early summer without a spring cereal as a cover crop. A cover crop would probably have lessened the competition by quick-establishing ryegrass.

Drought appeared to prevent DM overyielding in summer (Suppl. Fig. S 1), although diversity effects otherwise followed a similar pattern as in autumn (Suppl. Fig. S 2), which can be partly due to enhanced deep N uptake in summer drought (Hoekstra et al., 2015). Unexpectedly, ryegrass thrived in the drought summer, giving the highest DM yields and deep N recovery in pure stands and dominating in mixtures (Fig. 1).

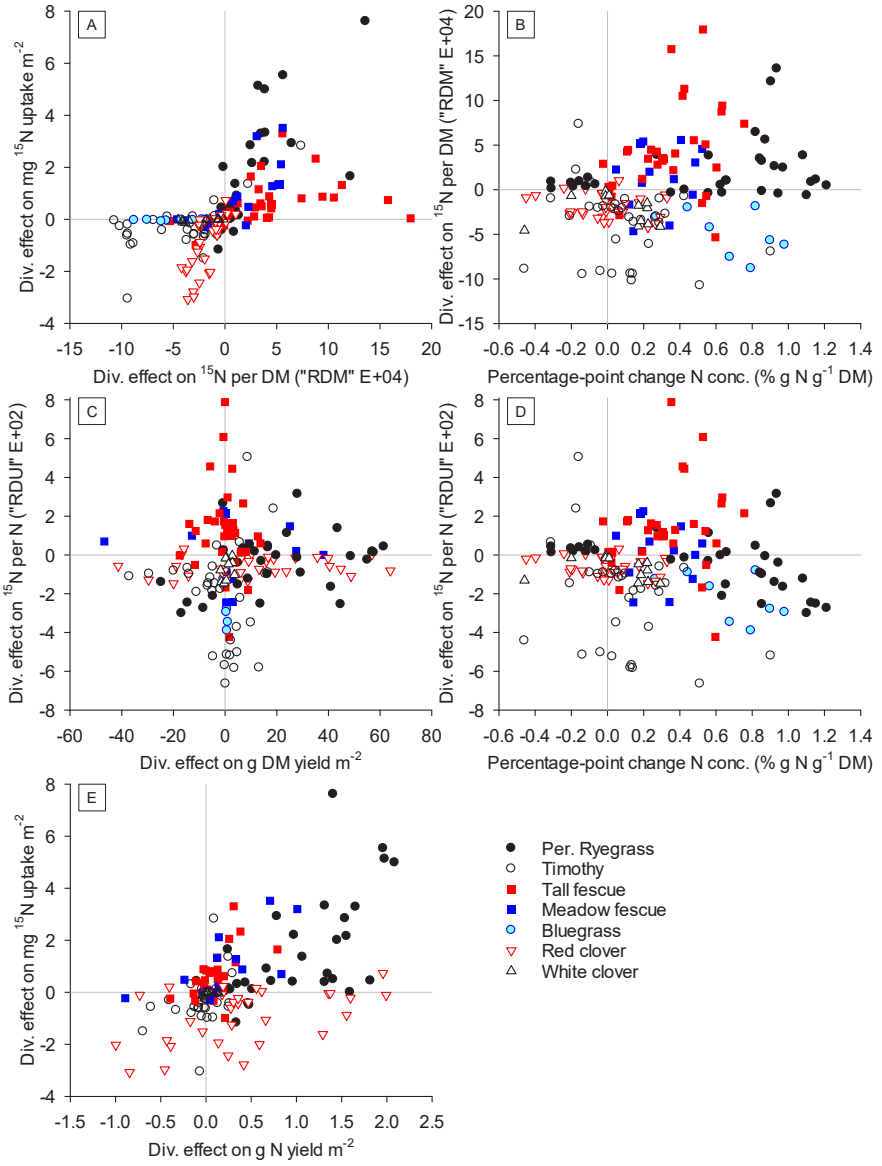


Figure 4: Relationships between selected diversity effects (*realized - expected* values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (Eqn. 6, 8; mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (Eqn. 7, 8; mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All observations shown from four replicates of each mixture at each of four harvests taken. The graphs per harvest are in Suppl. Fig. S 2.

4.2.2 Biologically-fixed nitrogen

The presence of clovers in the mixtures increased the N concentration in all grasses except timothy, and contributed 4-5 g N m^{-2} to the N yield of the mixtures, similar to results from Ergon et al. (2016) and Nyfeler et al. (2011). Nyfeler et al. (2011) found that neither fertilizer level nor proportion of clover to grasses altered the N concentration of clovers, but both of these factors had a positive effect on N concentration of grasses, especially of perennial ryegrass. They found that N overyielding and symbiotic N transfer to grasses were optimal with 40-60% legumes in the seed mixture at sowing, while Ergon et al. (2016) suggest that the optimal clover seeding proportions at the location of our experiment is closer to those used in this study (25% red clover in Mix 4 and 10% each of red and white clover in Mix 10), which translated to around 35-45% red clover in the DM yield in the third production year (Fig. 1). Red clover consistently recovered less deep N in mixtures than when grown in pure stands, indicating it relied increasingly on non-deep sources such as BNF. This is in partial contrast with Husse et al. (2017), who found that in mixture with other species red clover increased the uptake of ^{15}N at 30 cm depth relative to 3 cm depth.

4.2.3 Diversity in root behavior responses

Diversity effects on DM yield or on RDM can both contribute to ^{15}N (mg m^{-2}) overyielding. Plotting the positive or negative ^{15}N (mg m-2) diversity effects versus the diversity effects on RDM shows that changed deep N uptake patterns and not simply DM under- or overyielding contributed to the ^{15}N overyielding (Fig. 4 A). The positive (fescues and ryegrass) and negative (mainly red clover) diversity effects on ^{15}N uptake (mg m^{-2}) were linked respectively to positive or negative effects on RDM, which indicate a change in the N acquisition profile irrespective of diversity effects on plant vigor and effects of clover-associated BNF on the N nutrition of the grasses.

Ryegrass and the fescues showed increases in ^{15}N uptake and RDM while also increasing herbage N concentration and total N yield (Fig. 4 B, E). In ryegrass these positive diversity effects were combined with increased DM yield, though there was no linear relationship between growth vigor and deep N uptake (Fig. 4 C), while tall fescue's increased deep N uptake was despite showing no overall diversity effect on DM yield.

The contrasting behaviors of ryegrass and tall fescue were most prominent in the summer and autumn harvests (Suppl. Fig. S 2).

Tall fescue was the only species that increased deep N acquisition relatively more than total N, resulting in significant positive diversity effects on RDUI (Fig. 4 D, Suppl. Table S 2). This was most evident in summer and autumn, and it indicates a greater increase in uptake of deep-sourced N than of shallow-sourced N. Interestingly, this response could have not been predicted by the performance of tall fescue in pure stands, which in the summer and autumn harvests combined higher DM yield with lower RDM and RDUI than the other tall-growing grasses. Thus, competitive pressure from ryegrass and other species in the densest root zone stimulated tall fescue to increase deep root activity.

In ryegrass, decreased RDUI occurred together with increased RDM, indicating that ryegrass had a larger relative gain in total N than in ^{15}N. Mamolos et al. (1995) observed that following surface application of mineral N to a nutrient-poor permanent grassland, the most competitive species increased the activity of shallower (5 cm) rather than deeper (15 cm) roots. All other species in our experiment responded with significant reduction in RDUI, which in timothy and the clovers was independent of changes in N concentration, while in bluegrass it was the result of both increased N concentration and decreased RDM (Fig. 4 B).

Clovers and bluegrass showed negative diversity effects on deep N recovery. The fact that the clovers in mixture reduced their RDM relative to pure stands in summer and autumn (Suppl. Fig. S 2, H2 and H3) is in line with observations by Pirhofer-Walzl et al. (2013) who studied ^{15}N uptake from 80 and 120 cm depths by the deep-rooting legume alfalfa. However, a water-uptake study using ^{18}O found red clover sought water from deeper layers during drought in mixtures, but not in pure stands (Hoekstra et al., 2014). It could be that thick roots adapted for water transport are not equally effective for uptake of the less-mobile NH_4^+.

In our opinion a change in RDM when subjected to interaction with other species indicates a real effect on the distribution of root activity through the soil profile, which may or may not be combined with a change in root activity at a shallower depth. Thus a lack of positive effect on RDUI does not alone disprove the presence of a positive vertical niche effect.

4.2.4 N nutrition and deep N uptake

Within each harvest, the tall-growing grasses in pure stands generally had similar N nutritional statuses (Byers et al., Manuscript I of this thesis), but in mixtures they showed diverging diversity effects on N concentration, some positive and others nil or negative (Fig. 4 B). This implies these species varied in ability to exploit N niches in the mixtures. We did not estimate N nutritional status for individual species in the mixtures because it depends on the DM yield per area (Baadshaug and Lantinga, 2002), to which each component contributed only in part. However, comparing the diversity effects on N concentration to those on RDM and RDUI revealed different patterns of vertical niche differentiation (Fig. 4 B, D), as discussed below.

In the pure stands, N deficiency was combined with strong ^{15}N recovery in summer and autumn (Byers et al., Manuscript I of this thesis). The transgressive overyielding of ^{15}N recovery by Mix 4 in autumn was due to positive contributions by the fescues and ryegrass outweighing negative diversity effects in other species (Suppl. Fig. S 2, H3). Ryegrass, which tolerated the summer drought surprisingly well, increased its N concentration more than any other species both in the summer and autumn harvests, and in both mixtures, confirming its strong competitiveness for N (Maire et al., 2009). Tall and meadow fescue increased N concentration in mixtures by roughly half the amount (percentage-points) as ryegrass in summer and autumn (Suppl. Fig. S 2, H2 and H3).

If N deficiency drives deeper root foraging and uptake of mineral N below the ploughing depth, improved N status in grasses due to the presence of clovers could have reduced or made negative the diversity effects on ^{15}N uptake. This was the case of bluegrass, which proved to be able to compete for shallow, but not for deep-placed N. However, as mentioned, ryegrass, tall fescue and meadow fescue combined increased N concentration with increased deep N acquisition. We do not know if in the absence of clovers these positive diversity effects would have been even stronger.

We think that the stimulation of deeper N uptake in grasses as observed in our mixtures was conditional, both on adequate growth and competitive pressure for N from other species. Thus under moderate N fertilization and in presence of clovers, competition between grasses might stimulate a species to explore for N in deeper soil only if it has

sufficient herbage to support assimilation. This echoes the conclusion by Hoekstra et al. (2015) that including legumes reduces N limitation and allows for enhanced root exploration and nutrient uptake by other species.

We do not know how late autumn N reallocation to roots and overwintering might affect deep root N uptake. Any recommendations for seeding mixtures which capture N from deeper soil should also consider animal nutrition, so it is important that studies aim to optimize nitrogen use efficiency both from the standpoints of N loss prevention and protein yields.

4.3 Conclusions

As expected, the grass-clover mixtures used in our study overyielded in deep ^{15}N uptake as well as in herbage and N yields (Hypotheses 1 and 2). Furthermore, compared to single species in pure stands, mixtures provided more evenly-distributed yields with a more even protein content throughout the growing season, and provided the best combination of annual DM yields with very high protein yields and deep N recovery in one single sward. This confirms the agronomic and environmental advantage of using well-adapted mixtures for forage production.

The overyielding in deep N acquisition increased from weak in the spring harvest to transgressive overyielding in the autumn. Thus the mixtures recovered more deep N late-season than the best-performing grass in pure stands, and left little mineral N in the soil despite high protein yields. This has positive implications for reducing N dissipation and emissions of the greenhouse gas nitrous oxide while increasing high-quality forage production.

Grasses exhibited vertical niche differentiation: ryegrass and the fescues increased uptake of deep-placed $^{15}NH_4^+$ in mixtures, while timothy and bluegrass decreased it. This partially refutes Hypothesis 3. The species' ability to recover deep N in pure stands was thus not sufficient to predict their behavior in mixtures. For example, timothy in pure stands was among the best grasses at utilizing deep N, but consistently reduced this ability in mixtures; tall fescue, which did not show superior ability for acquiring deep N in pure stand, consistently increased deep N acquisition in mixture. Overall, we believe that ryegrass benefits the N utilization throughout the whole soil profile,

including deep-placed N, while contributing to high forage quality, though its dominance may suppress persistence of other species. Tall fescue, while it should not comprise a large share of mixtures due to moderate forage quality, can likely improve sward uptake of deep N, especially if activated by diversity effects.

If improved N availability due to the presence of clovers reduced a positive diversity effect on deep N uptake by grasses (Hypothesis 4), this effect was not large enough to prevent overyielding in deep N uptake in ryegrass, tall fescue and meadow fescue. Clovers decreased deep N uptake in mixtures relative to pure stands, as expected (Hypothesis 5).

We think that stimulation of deeper N uptake in grasses as observed in our mixtures was conditional both on adequate growth and competitive pressure for N from other species. Further studies are needed to explore this, as well as how late-season N reallocation to roots and winter survival affect deep N recovery.

References

Baadshaug, O.H., Lantinga, E.A., 2002. ENGNOR, a Grassland Crop Growth Model for High Latitudes. Technical Documentation. (Reports from AUN. Department of Plant Sciences No. 2/2002). Agricultural University of Norway.

Bleken, M.A., Rittl, T.F., Nadeem, S., Hansen, S., 2022. Roots and other residues from leys with or without red clover: Quality and effects on N_2O emission factors in a partly frozen soil following autumn ploughing. Science of The Total Environment 831, 154582. https://doi.org/10.1016/j.scitotenv.2022.154582

Byers, E., Bleken, M.A., Dörsch, P., 2021. Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH. Environ. Res. Commun. 3, 015001. https://doi.org/10.1088/2515-7620/abd623

Byers, E., Dörsch, P., Eich-Greatorex, S., Bleken, M.A. (2023) Deep N acquisition in hemiboreal cultivated grasslands: I. Species in pure stands. Manuscript I of this Thesis. Norwegian University of Life Sciences.

Cahill, J.F., McNickle, G.G., 2011. The Behavioral Ecology of Nutrient Foraging by Plants. Annual Review of Ecology, Evolution, and Systematics 42, 289–311. https://doi.org/10.1146/annurev-ecolsys-102710-145006

Cardinale, B.J., Wright, J.P., Cadotte, M.W., Carroll, I.T., Hector, A., Srivastava, D.S., Loreau, M., Weis, J.J., 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. Proceedings of the National Academy of Sciences 104, 18123–18128. https://doi.org/10.1073/pnas.0709069104

Chen, S.-M., Lin, S., Loges, R., Reinsch, T., Hasler, M., Taube, F., 2016. Independence of seasonal patterns of root functional traits and rooting strategy of a grass-clover sward from sward age and slurry application. Grass Forage Sci 71, 607–621. https://doi.org/10.1111/gfs.12222

Dhamala, N.R., Rasmussen, J., Carlsson, G., Søegaard, K., Eriksen, J., 2017. N transfer in three-species grass-clover mixtures with chicory, ribwort plantain or caraway. Plant and Soil 413, 217–230. https://doi.org/10.1007/s11104-016-3088-6

Ergon, Å., Kirwan, L., Bleken, M.A., Skjelvåg, A.O., Collins, R.P., Rognli, O.A., 2016. Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. Grass and Forage Science 71, 667–682. https://doi.org/10.1111/gfs.12250

Fargione, J., Tilman, D., Dybzinski, R., Lambers, J.H.R., Clark, C., Harpole, W.S., Knops, J.M.H., Reich, P.B., Loreau, M., 2007. From selection to complementarity: shifts in the causes of biodiversity–productivity relationships in a long-term biodiversity experiment. Proceedings of the Royal Society B: Biological Sciences 274, 871–876. https://doi.org/10.1098/rspb.2006.0351

Finn, J.A., Kirwan, L., Connolly, J., Sebastià, M.T., Helgadottir, A., Baadshaug, O.H., Bélanger, G., Black, A., Brophy, C., Collins, R.P., Čop, J., Dalmannsdóttir, S., Delgado, I., Elgersma, A., Fothergill, M., Frankow-Lindberg, B.E., Ghesquiere, A., Golinska, B., Golinski, P., Grieu, P., Gustavsson, A.-M., Höglind, M., Huguenin-Elie, O., Jørgensen, M., Kadziuliene, Z., Kurki, P., Llurba, R., Lunnan, T., Porqueddu, C., Suter, M., Thumm, U., Lüscher, A., 2013. Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. Journal of Applied Ecology 50, 365–375. https://doi.org/10.1111/1365-2664.12041

Hernandez, P., Picon-Cochard, C., 2016. Presence of Trifolium repens Promotes Complementarity of Water Use and N Facilitation in Diverse Grass Mixtures. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.00538

Hodge, A., 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytologist 162, 9–24. https://doi.org/10.1111/j.1469-8137.2004.01015.x

Hoekstra, N.J., Finn, J.A., Hofer, D., Lüscher, A., 2014. The effect of drought and interspecific interactions on depth of water uptake in deep- and shallow-rooting grassland species as determined by $\delta\ ^{18}O$ natural abundance. Biogeosciences 11, 4493–4506. https://doi.org/10.5194/bg-11-4493-2014

Hoekstra, N.J., Suter, M., Finn, J.A., Husse, S., Lüscher, A., 2015. Do belowground vertical niche differences between deep- and shallow-rooted species enhance resource uptake and drought resistance in grassland mixtures? Plant and Soil 394, 21–34. https://doi.org/10.1007/s11104-014-2352-x

Hooper, D.U., 1998. The role of complementarity and competition in ecosystem responses to variation in plant diversity. Ecology 79, 704–719. https://doi.org/10.1890/0012-9658(1998)079[0704:TROCAC]2.0.CO;2

Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A.J., Vandermeer, J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75, 3–35. https://doi.org/10.1890/04-0922

Husse, S., Huguenin-Elie, O., Buchmann, N., Lüscher, A., 2016. Larger yields of mixtures than monocultures of cultivated grassland species match with asynchrony in shoot growth among species but not with increased light interception. Field Crops Research 194, 1–11. https://doi.org/10.1016/j.fcr.2016.04.021

Husse, S., Lüscher, A., Buchmann, N., Hoekstra, N.J., Huguenin-Elie, O., 2017. Effects of mixing forage species contrasting in vertical and temporal nutrient capture on nutrient yields and fertilizer recovery in productive grasslands. Plant Soil 420, 505–521. https://doi.org/10.1007/s11104-017-3372-0

IUSS Working Group WRB, 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps, World Soil Resources Reports No. 106. FAO, Rome.

Jing, J., Søegaard, K., Cong, W.-F., Eriksen, J., 2017. Species Diversity Effects on Productivity, Persistence and Quality of Multispecies Swards in a Four-Year Experiment. PLOS ONE 12, e0169208. https://doi.org/10.1371/journal.pone.0169208

Kirwan, L., Connolly, J., Finn, J.A., Brophy, C., Lüscher, A., Nyfeler, D., Sebastià, M.-T., 2009. Diversity–interaction modeling: estimating contributions of species identities and interactions to ecosystem function. Ecology 90, 2032–2038. https://doi.org/10.1890/08-1684.1

Kristensen, H.L., Thorup-Kristensen, K., 2004. Root Growth and Nitrate Uptake of Three Different Catch Crops in Deep Soil Layers. Soil Science Society of America Journal 68, 529. https://doi.org/10.2136/sssaj2004.0529

Lüscher, A., Finn, J.A., Connolly, J., Sebastià, M.T., Collins, R., Fothergill, M., Porqueddu, C., Brophy, C., Huguenin-Elie, O., Kirwan, L., Nyfeler, D., Helgadóttir, A., 2008. Benefits of sward diversity for agricultural grasslands. Biodiversity 9, 29–32. https://doi.org/10.1080/14888386.2008.9712877

Lüscher, A., Mueller-Harvey, I., Soussana, J.F., Rees, R.M., Peyraud, J.L., 2014. Potential of legume-based grassland-livestock systems in Europe: a review. Grass and Forage Science 69, 206–228. https://doi.org/10.1111/gfs.12124

Maire, V., Gross, N., da Silveira Pontes, L., Picon-Cochard, C., Soussana, J.-F., 2009. Trade-off between root nitrogen acquisition and shoot nitrogen utilization across 13 co-occurring pasture grass species. Functional Ecology 23, 668–679. https://doi.org/10.1111/j.1365-2435.2009.01557.x

Malcolm, B.J., Moir, J.L., Cameron, K.C., Di, H.J., Edwards, G.R., 2015. Influence of plant growth and root architecture of Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) on N recovery during winter. Grass and Forage Science 70, 600–610. https://doi.org/10.1111/gfs.12157

Mamolos, A.P., Elisseou, G.K., Veresoglou, D.S., 1995. Depth of Root Activity of Coexisting Grassland Species in Relation to N and P Additions, Measured Using Nonradioactive Tracers. The Journal of Ecology 83, 643. https://doi.org/10.2307/2261632

Mamolos, A.P., Veresoglou, D.S., 2000. Patterns of root activity and responses of species to nutrients in vegetation of fertile alluvial soil. Plant Ecology 148, 245–253. https://doi.org/10.1023/A:1009890630391

Milovanović, J., Eich-Greatorex, S., Krogstad, T., Rakic, V., Rajic, N., 2015. The use in grass production of clinoptilolite as an ammonia adsorbent and a nitrogen carrier. Journal of the Serbian Chemical Society 80, 1203–1214. https://doi.org/10.2298/JSC150317042M

Mommer, L., Van Ruijven, J., De Caluwe, H., Smit-Tiekstra, A.E., Wagemaker, C.A.M., Joop Ouborg, N., Bögemann, G.M., Van Der Weerden, G.M., Berendse, F., De Kroon, H., 2010. Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species: Below-ground species distributions in a biodiversity experiment. Journal of Ecology 98, 1117–1127. https://doi.org/10.1111/j.1365-2745.2010.01702.x

Nyfeler, D., Huguenin-Elie, O., Suter, M., Frossard, E., Lüscher, A., 2011. Grass–legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources. Agriculture, Ecosystems & Environment 140, 155–163. https://doi.org/10.1016/j.agee.2010.11.022

Østrem, L., Rapacz, M., Larsen, A., Dalmannsdottir, S., Jørgensen, M., 2015. Influences of growth cessation and photoacclimation on winter survival of non-native Lolium–Festuca grasses in high-latitude regions. Environmental and Experimental Botany 111, 21–31. https://doi.org/10.1016/j.envexpbot.2014.10.008

Pirhofer-Walzl, K., Eriksen, J., Rasmussen, J., Høgh-Jensen, H., Søegaard, K., Rasmussen, J., 2013. Effect of four plant species on soil ^{15}N-access and herbage yield in temporary agricultural grasslands. Plant and Soil 371, 313–325. https://doi.org/10.1007/s11104-013-1694-0

Ravenek, J.M., Bessler, H., Engels, C., Scherer-Lorenzen, M., Gessler, A., Gockele, A., De Luca, E., Temperton, V.M., Ebeling, A., Roscher, C., Schmid, B., Weisser, W.W., Wirth, C., de Kroon, H., Weigelt, A., Mommer, L., 2014. Long-term study of root biomass in a biodiversity experiment reveals shifts in diversity effects over time. Oikos 123, 1528–1536. https://doi.org/10.1111/oik.01502

Reinsch, T., Loges, R., Kluß, C., Taube, F., 2018. Renovation and conversion of permanent grass-clover swards to pasture or crops: Effects on annual N_2O emissions in the year after ploughing. Soil and Tillage Research 175, 119–129. https://doi.org/10.1016/j.still.2017.08.009

Robinson, D., Hodge, A., Griffiths, B.S., Fitter, A.H., 1999. Plant root proliferation in nitrogen–rich patches confers competitive advantage. Proceedings of the Royal Society of London. Series B: Biological Sciences 266, 431–435. https://doi.org/10.1098/rspb.1999.0656

Schmid, B., Hector, A., Saha, P., Loreau, M., 2008. Biodiversity effects and transgressive overyielding. Journal of Plant Ecology 1, 95–102. https://doi.org/10.1093/jpe/rtn011

von Felten, S., Niklaus, P.A., Scherer-Lorenzen, M., Hector, A., Buchmann, N., 2012. Do grassland plant communities profit from N partitioning by soil depth? Ecology 93, 2386–2396. https://doi.org/10.1890/11-1439.1

von Felten, S., Schmid, B., 2008. Complementarity among species in horizontal versus vertical rooting space. Journal of Plant Ecology 1, 33–41. https://doi.org/10.1093/jpe/rtm006

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2021. Meteorologiske data for Ås 2020. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2018. Meteorologiske data for Ås 2017. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Wolff, M., Thue-Hansen, V., Grimenes, A.A., 2017. Meteorologiske data for Ås 2016. Feltstasjon for bioklimatiske studier, Sørås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet. Available online: https://www.nmbu.no/fakultet/realtek/laboratorier/bioklim/meteorologiske-data

Deep N acquisition in hemiboreal cultivated grasslands: II. Niche and overyielding effects in mixtures

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina A. Bleken

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Supplementary materials

Table S 1: Mineral N (sum of NO^{3-} and NH_4^+ N) by depth in soil **A)** removed when placing ^{15}N-labeled clinoptilolite, and **B)** sampled at the end of the growing season 2017 in mixtures and pure stands, which are presented in Byers et al. (Manuscript I of this thesis). Means of 4 replicates (±SE). *Post hoc* Tukey grouping ($p<0.05$) for each sampling and date combination.

A) Sampled 19 April 2017 at time of ^{15}N label placement

	Min-N g m^{-2}, 0-20 cm depth	Min-N g m^{-2}, 20-30 cm depth	Min-N g m^{-2}, 30-43 cm depth	**Whole profile: 0-43 cm depth**
Mix 4	0.70 (±0.02) BC	0.55 (±0.12) AB	0.52 (±0.01) B	1.77 (±0.13) B
Mix 10	0.77 (±0.10) BC	0.39 (±0.08) B	0.43 (±0.07) B	1.59 (±0.11) B
Pure stands				
Per. Ryegrass	1.08 (±0.19) ABC	0.33 (±0.05) B	0.55 (±0.05) B	1.96 (±0.16) B
Timothy	0.52 (±0.15) BC	0.37 (±0.06) B	0.40 (±0.07) B	1.30 (±0.17) B
Tall fescue	0.54 (±0.12) BC	0.38 (±0.06) B	0.33 (±0.03) B	1.25 (±0.10) B
Mea. fescue	0.77 (±0.19) BC	0.28 (±0.05) B	0.42 (±0.02) B	1.48 (±0.20) B
Bluegrass	0.40 (±0.11) C	0.26 (±0.04) B	0.43 (±0.10) B	1.09 (±0.21) B
Red clover	1.54 (±0.39) AB	0.87 (±0.17) A	1.15 (±0.19) A	3.56 (±0.53) A
White clover	1.93 (±0.43) A	0.70 (±0.14) AB	1.24 (±0.12) A	3.87 (±0.34) A

B) Sampled 30 October 2017 at end of first growing season after ^{15}N label placement

	Min-N g m^{-2}, 0-20 cm depth	Min-N g m^{-2}, 20-40 cm depth	Min-N g m^{-2}, 40-60 cm depth	**Whole profile: 0-60 cm depth**
Mix 4	0.73 (±0.10) B	0.24 (±0.05) B	0.28 (±0.14) AB	1.25 (±0.16) B
Pure stands				
Per. Ryegrass	0.67 (±0.04) BC	0.30 (±0.04) B	0.16 (±0.02) B	1.13 (±0.06) B
Tall fescue	0.45 (±0.03) C	0.21 (±0.04) B	0.14 (±0.04) B	0.81 (±0.08) B
Red clover	1.40 (±0.06) A	1.04 (±0.11) A	0.72 (±0.17) A	3.15 (±0.33) A

Table S 2 (A, H1): Component species results in both mixtures combined (top line for per. ryegrass, timothy, tall fescue and red clover), Mix 4, and Mix 10. Mean of 4 replicates (±SE). Significance of two-sided t-test shown; values above p=0.15 are marked ns; values under p=0.15 are bolded. Overyielding of DM, N and ^{15}N in bluegrass and white clover are based on small samples and should be interpreted with caution even when significant.

A) H1: 6 June 2017	Realized yields			Contrib. to overyielding (±SE) as % incr. from expected value			Diversity effect (Realized - Expected) on:		
	DM yield (g DM m^{-2})	N yield (g N m^{-2})	^{15}N herbage uptake (mg ^{15}N m^{-2})	DM yield	N yield	^{15}N herbage uptake	N conc., Pct. points g N g^{-1} DM	RDM (E+04)	RDUI (E+02)
Per. Ryegrass						**Pooled:**	**+0.55 (±0.03), p<0.05**	+1.03 (±0.23), ns	+0.19 (±0.09), ns
Mix 4	90.6 (±11.0)	2.51 (±0.28)	2.51 (±0.36)	**+55.3% (±9.2%), p=0.06**	**+82.4% (±10.5%), p<0.05**	**+173.6% (±39.1%), p=0.11**	**+0.46 (±0.04), p<0.05**	**+2.11 (±0.53), p=0.14**	+0.60 (±0.19), ns
Mix 10	38.0 (±6.2)	1.10 (±0.17)	0.6 (±0.10)	**+78.3% (±11.3%), p<0.05**	**+119.4% (±15.8%), p<0.05**	**+74.6% (±14.9%), p=0.09**	**+0.64 (±0.06), p<0.05**	-0.06 (±0.11), ns	**-0.21 (±0.04), p=0.09**
Timothy						**Pooled:**	**+0.19 (±0.02), p<0.05**	**-2.76 (±0.12), p<0.05**	**-1.48 (±0.05), p<0.05**
Mix 4	22.8 (±2.9)	0.49 (±0.06)	0.2 (±0.05)	**-39.7% (±9.9%), p=0.14**	-34.3% (±10.1%), ns	**-83.9% (±12.2%), p<0.05**	**+0.20 (±0.02), p<0.05**	**-3.09 (±0.19), p<0.05**	**-1.61 (±0.09), p<0.05**
Mix 10	30.2 (±5.1)	0.67 (±0.12)	0.4 (±0.11)	**-18.2% (±3.8%), p=0.09**	-7.7% (±2.2%), ns	**-60.4% (±8.9%), p<0.05**	+0.18 (±0.07), ns	**-2.44 (±0.29), p<0.05**	**-1.34 (±0.12), p<0.05**
Tall fescue						**Pooled:**	**+0.36 (±0.04), p<0.05**	**+2.29 (±0.42), p=0.09**	**+0.65 (±0.14), p=0.13**
Mix 4	13.3 (±2.8)	0.37 (±0.08)	0.5 (±0.12)	-20.8% (±8.2%), ns	-1.7% (±5.4%), ns	+56.9% (±39.9%), ns	**+0.46 (±0.06), p<0.05**	**+3.45 (±0.84), p=0.13**	+1.04 (±0.29), ns
Mix 10	86.4 (±4.0)	2.11 (±0.11)	2.0 (±0.32)	+6.7% (±3.1%), ns	+15.6% (±8.1%), ns	+37.9% (±23.6%), ns	+0.22 (±0.11), ns	+0.73 (±0.55), ns	+0.13 (±0.16), ns
Red clover						**Pooled:**	-0.11 (±0.03), ns	**-0.52 (±0.06), p<0.05**	**-0.13 (±0.02), p<0.05**
Mix 4	113.4 (±8.4)	4.17 (±0.29)	0.8 (±0.04)	+6.3% (±4.2%), ns	+1.2% (±3.2%), ns	-20.2% (±8.6%), ns	**-0.19 (±0.04), p=0.13**	-0.27 (±0.12), ns	-0.06 (±0.03), ns
Mix 10	106.5 (±8.3)	4.11 (±0.37)	0.6 (±0.09)	**+32.1% (±4.5%), p<0.05**	**+32.1% (±6.6%), p=0.09**	**-30.0% (±6.1%), p=0.09**	-0.04 (±0.08), ns	**-0.77 (±0.09), p<0.05**	**-0.20 (±0.02), p<0.05**
Mea. fescue Mix 10 only	70.5 (±30.5)	1.60 (±0.73)	1.9 (±1.12)	-0.3% (±0.7%), ns	+16.0% (±9.2%), ns	+18.1% (±25.9%), ns	+0.24 (±0.09), ns	-0.24 (±1.01), ns	-0.33 (±0.40), ns
Bluegrass Mix 10 only	1.2 (±0.7)	0.04 (±0.02)	0.0 (±0.00)	+22.8% (±13.3%), ns	+59.1% (±38.6%), ns	-75.8% (±40.4%), ns	+0.43 (±0.27), ns	**-2.04 (±0.14), p=0.06**	**-0.84 (±0.04), p<0.05**
White clover Mix 10 only	1.8 (±0.6)	0.08 (±0.03)	0.0 (±0.00)	+221.8% (±80.1%), ns	+220.9% (±80.9%), ns	+84.9% (±79.9%), ns	-0.07 (±0.04), ns	**-0.51 (±0.10), p=0.11**	**-0.11 (±0.02), p=0.11**

Table S 2 (B, H2): Component species results in both mixtures combined (top line for per. ryegrass, timothy, tall fescue and red clover), Mix 4, and Mix 10. Mean of 4 replicates (±SE). Significance of two-sided t-test shown; values above p=0.15 are marked ns; values under p=0.15 are bolded. Overyielding of DM, N and ^{15}N in bluegrass and white clover are based on small samples and should be interpreted with caution even when significant.

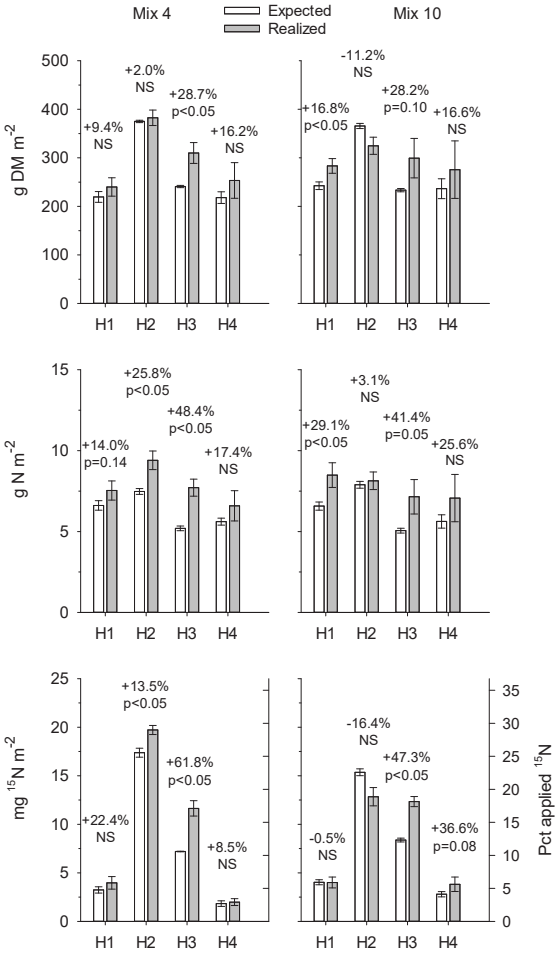
B) H2: 19 July 2017	Realized yields			Contrib. to overyielding (±SE) as % incr. from expected value			Diversity effect (Realized - Expected) on:		
	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	^{15}N herbage uptake (mg ^{15}N m^{-2})	DM yield	N yield	^{15}N herbage uptake	N conc., Pct. points g N g^{-1} DM	RDM (E+04)	RDUI (E+02)
Per. Ryegrass						**Pooled:**	**+0.95 (±0.03), p<0.05**	**+1.39 (±0.21), p<0.05**	**-1.93 (±0.09), p<0.05**
Mix 4	186.3 (±6.3)	4.38 (±0.20)	14.2 (±0.68)	+0.7% (±2.4%), ns	**+61.6% (±4.3%), p<0.05**	**+31.6% (±4.5%), p<0.05**	**+0.88 (±0.04), p<0.05**	**+2.68 (±0.31), p<0.05**	**-1.28 (±0.06), p<0.05**
Mix 10	85.4 (±10.0)	2.13 (±0.28)	5.1 (±0.62)	**-11.5% (±1.4%), p<0.05**	**+50.8% (±10.4%), p=0.09**	**-9.1% (±2.1%), p=0.12**	**+1.02 (±0.07), p<0.05**	+0.10 (±0.17), ns	**-2.58 (±0.10), p<0.05**
Timothy						**Pooled:**	-0.09 (±0.03), ns	**-5.84 (±0.77), p<0.05**	**-2.94 (±0.48), p=0.07**
Mix 4	24.7 (±3.7)	0.44 (±0.06)	1.7 (±0.65)	**+23.1% (±4.0%), p=0.06**	**+20.3% (±3.8%), p=0.07**	+13.8% (±29.7%), ns	-0.03 (±0.04), ns	-4.32 (±1.99), ns	-2.21 (±1.22), ns
Mix 10	29.3 (±5.3)	0.47 (±0.09)	1.1 (±0.38)	+2.7% (±3.8%), ns	**-7.5% (±1.4%), p=0.08**	-49.0% (±16.8%), ns	-0.15 (±0.07), ns	**-7.35 (±1.07), p<0.05**	**-3.67 (±0.73), p=0.09**
Tall fescue						**Pooled:**	**+0.30 (±0.03), p<0.05**	**+3.16 (±0.55), p=0.08**	+0.87 (±0.25), ns
Mix 4	40.9 (±6.5)	0.75 (±0.11)	2.0 (±0.07)	**+11.8% (±2.8%), p=0.12**	**+21.0% (±4.1%), p=0.08**	+22.1% (±15.0%), ns	+0.16 (±0.05), ns	+3.15 (±1.49), ns	+1.14 (±0.66), ns
Mix 10	63.7 (±11.3)	1.35 (±0.24)	4.7 (±0.89)	-7.9% (±3.0%), ns	**+15.2% (±2.7%), p=0.07**	**+54.7% (±11.5%), p=0.10**	**+0.44 (±0.04), p<0.05**	**+3.16 (±0.81), p=0.14**	+0.61 (±0.38), ns
Red clover						**Pooled:**	**+0.09 (±0.01), p=0.05**	**-2.00 (±0.18), p<0.05**	**-0.74 (±0.06), p<0.05**
Mix 4	130.5 (±8.4)	3.84 (±0.26)	1.8 (±0.22)	-2.1% (±1.7%), ns	+1.4% (±2.4%), ns	**-47.0% (±9.6%), p=0.09**	+0.11 (±0.03), ns	-1.61 (±0.46), ns	-0.61 (±0.16), ns
Mix 10	127.9 (±8.0)	3.70 (±0.23)	1.5 (±0.27)	**-16.9% (±2.0%), p<0.05**	**-15.4% (±1.7%), p<0.05**	**-63.4% (±3.5%), p<0.05**	+0.06 (±0.02), ns	**-2.39 (±0.27), p<0.05**	**-0.88 (±0.09), p<0.05**
Mea. fescue Mix 10 only	8.4 (±2.2)	0.21 (±0.06)	0.5 (±0.15)	**+37.0% (±8.8%), p=0.14**	+63.3% (±16.8%), ns	+14.4% (±10.7%), ns	**+0.32 (±0.06), p=0.08**	**-3.07 (±0.73), p=0.14**	**-2.04 (±0.23), p<0.05**
Bluegrass Mix 10 only	2.2 (±0.1)	0.07 (±0.00)	0.0 (±0.00)	**+38.4% (±5.9%), p=0.14**	**+100.8% (±1.2%), p<0.05**	**-96.0% (±16.3%), p=0.15**	**+0.94 (±0.03), p<0.05**	**-5.92 (±0.17), p<0.05**	**-2.87 (±0.06), p<0.05**
White clover Mix 10 only	6.2 (±1.6)	0.19 (±0.05)	0.1 (±0.03)	**-14.0% (±2.4%), p=0.06**	**-7.6% (±1.5%), p=0.08**	**-70.6% (±10.3%), p<0.05**	**+0.23 (±0.01), p<0.05**	**-3.52 (±0.25), p<0.05**	**-1.27 (±0.08), p<0.05**

Table S 2 (C, H3): Component species results in both mixtures combined (top line for per. ryegrass, timothy, tall fescue and red clover), Mix 4, and Mix 10. Mean of 4 replicates (±SE). Significance of two-sided t-test shown; values above p=0.15 are marked ns; values under p=0.15 are bolded. Overyielding of DM, N and ^{15}N in bluegrass and white clover are based on small samples and should be interpreted with caution even when significant.

C) H3: 18 Sept. 2017	Realized yields			Contrib. to overyielding (±SE) as % incr. from expected value			Diversity effect (Realized - Expected) on:		
	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	^{15}N herbage uptake (mg ^{15}N m^{-2})	DM yield	N yield	^{15}N herbage uptake	N conc., Pct. points g N g^{-1} DM	RDM (E+04)	RDUI (E+02)
Per. Ryegrass						**Pooled:**	**+0.92 (±0.01), p<0.05**	**+5.60 (±0.62), p<0.05**	-0.17 (±0.27), ns
Mix 4	124.5 (±6.3)	2.88 (±0.13)	8.4 (±0.60)	**+41.9% (±2.8%), p<0.05**	**+131.9% (±4.8%), p<0.05**	**+134.7% (±15.4%), p<0.05**	**+0.91 (±0.01), p<0.05**	**+6.29 (±1.26), p=0.09**	+0.13 (±0.53), ns
Mix 10	57.9 (±7.5)	1.34 (±0.16)	3.7 (±0.34)	**+47.1% (±12.1%), p=0.15**	**+141.1% (±22.5%), p=0.05**	**+100.8% (±11.3%), p<0.05**	**+0.93 (±0.04), p<0.05**	+4.90 (±1.40), ns	-0.48 (±0.63), ns
Timothy						**Pooled:**	**+0.22 (±0.04), p=0.10**	**-5.49 (±0.57), p<0.05**	**-3.47 (±0.38), p<0.05**
Mix 4	15.6 (±3.3)	0.26 (±0.04)	0.7 (±0.25)	**+94.1% (±23.9%), p=0.14**	**+95.3% (±15.9%), p=0.06**	+78.2% (±44.4%), ns	+0.25 (±0.11), ns	-4.30 (±1.27), ns	-2.71 (±0.94), ns
Mix 10	20.9 (±4.0)	0.36 (±0.07)	0.7 (±0.22)	**+86.9% (±15.1%), p=0.06**	**+98.2% (±17.1%), p=0.06**	+9.6% (±20.7%), ns	+0.20 (±0.05), ns	**-6.68 (±1.09), p=0.05**	**-4.22 (±0.64), p<0.05**
Tall fescue						**Pooled:**	**+0.44 (±0.02), p<0.05**	**+6.18 (±0.80), p<0.05**	**+2.12 (±0.43), p=0.13**
Mix 4	29.2 (±3.9)	0.50 (±0.07)	1.6 (±0.14)	+19.3% (±5.7%), ns	**+46.5% (±8.8%), p=0.08**	**+92.9% (±8.8%), p<0.05**	**+0.32 (±0.01), p<0.05**	**+6.50 (±1.55), p=0.13**	+2.75 (±0.86), ns
Mix 10	21.6 (±4.1)	0.43 (±0.08)	1.8 (±0.39)	+4.5% (±6.3%), ns	+48.3% (±14.5%), ns	**+125.7% (±31.4%), p=0.14**	**+0.57 (±0.03), p<0.05**	+5.85 (±1.87), ns	+1.49 (±0.98), ns
Red clover						**Pooled:**	-0.01 (±0.02), ns	**-3.13 (±0.08), p<0.05**	**-1.08 (±0.03), p<0.05**
Mix 4	140.7 (±9.0)	4.07 (±0.26)	1.0 (±0.10)	+16.6% (±4.5%), ns	**+17.0% (±4.3%), p=0.14**	**-60.3% (±4.6%), p<0.05**	+0.02 (±0.04), ns	**-3.02 (±0.15), p<0.05**	**-1.05 (±0.05), p<0.05**
Mix 10	125.4 (±13.2)	3.51 (±0.34)	1.0 (±0.22)	+16.0% (±8.1%), ns	+12.4% (±6.7%), ns	**-59.6% (±5.1%), p<0.05**	-0.04 (±0.05), ns	**-3.24 (±0.21), p<0.05**	**-1.11 (±0.08), p<0.05**
Mea. fescue Mix 10 only	66.4 (±5.3)	1.34 (±0.10)	5.0 (±0.42)	**+37.0% (±8.8%), p=0.13**	**+68.8% (±12.2%), p=0.07**	**+100.8% (±10.2%), p<0.05**	**+0.40 (±0.04), p<0.05**	**+4.66 (±0.29), p<0.05**	**+1.08 (±0.25), p=0.12**
Bluegrass Mix 10 only	1.6 (±0.1)	0.05 (±0.00)	0.0 (±0.00)	**+129.8% (±15.4%), p=0.11**	**+205.7% (±15.5%), p=0.07**	-68.3% (±16.5%), ns	**+0.73 (±0.04), p=0.05**	**-8.16 (±0.45), p<0.05**	**-3.67 (±0.15), p<0.05**
White clover Mix 10 only	4.1 (±2.1)	0.15 (±0.08)	0.0 (±0.02)	+98.6% (±53.9%), ns	+104.7% (±63.5%), ns	-52.7% (±13.4%), ns	-0.14 (±0.23), ns	**-4.03 (±0.38), p=0.08**	**-1.16 (±0.10), p=0.08**

Table S 2 (D, H4): Component species results in both mixtures combined (top line for per. ryegrass, timothy, tall fescue and red clover), Mix 4, and Mix 10. Mean of 4 replicates (±SE). Significance of two-sided t-test shown; values above p=0.15 are marked ns; values under p=0.15 are bolded. Overyielding of DM, N and ^{15}N in bluegrass and white clover are based on small samples and should be interpreted with caution even when significant.

D) H4: 30 May 2018	Realized yields			Contrib. to overyielding (±SE) as % incr. from expected value			Diversity effect (Realized - Expected) on:		
	DM Yield (g DM m^{-2})	N yield (g N m^{-2})	^{15}N herbage uptake (mg ^{15}N m^{-2})	DM yield	N yield	^{15}N herbage uptake	N conc., Pct. points g N g^{-1} DM	RDM (E+04)	RDUI (E+02)
Per. Ryegrass						**Pooled:**	**-0.18 (±0.01), p<0.05**	**+0.69 (±0.05), p<0.05**	**+0.30 (±0.02), p<0.05**
Mix 4	132.7 (±10.1)	3.45 (±0.27)	1.0 (±0.06)	**+36.5% (±7.1%), p=0.08**	**+27.7% (±6.9%), p=0.14**	**+103.0% (±8.7%), p<0.05**	**-0.20 (±0.02), p<0.05**	**+0.76 (±0.06), p<0.05**	**+0.34 (±0.03), p<0.05**
Mix 10	40.2 (±9.4)	1.03 (±0.23)	0.3 (±0.05)	+94.5% (±31.2%), ns	+78.7% (±25.6%), ns	**+144.7% (±31.9%), p=0.11**	**-0.17 (±0.03), p=0.05**	**+0.62 (±0.13), p=0.10**	**+0.27 (±0.04), p=0.06**
Timothy						**Pooled:**	+0.02 (±0.02), ns	**-1.49 (±0.13), p<0.05**	**-0.73 (±0.06), p<0.05**
Mix 4	44.2 (±8.9)	0.93 (±0.18)	0.2 (±0.03)	**-23.2% (±6.0%), p=0.15**	-22.6% (±5.9%), ns	**-69.4% (±10.7%), p<0.05**	+0.08 (±0.03), ns	**-1.62 (±0.11), p<0.05**	**-0.82 (±0.04), p<0.05**
Mix 10	64.3 (±10.5)	1.29 (±0.21)	0.5 (±0.16)	-2.4% (±4.9%), ns	-6.4% (±4.5%), ns	-30.5% (±14.2%), ns	-0.04 (±0.02), ns	-1.37 (±0.38), ns	-0.64 (±0.19), ns
Tall fescue						**Pooled:**	**+0.22 (±0.02), p<0.05**	**+4.71 (±0.71), p=0.05**	**+1.66 (±0.24), p<0.05**
Mix 4	36.3 (±6.7)	0.84 (±0.15)	0.7 (±0.11)	-10.4% (±6.6%), ns	-8.9% (±6.6%), ns	+19.2% (±12.6%), ns	+0.16 (±0.06), ns	+5.67 (±2.11), ns	+2.01 (±0.71), ns
Mix 10	25.1 (±6.2)	0.62 (±0.15)	0.8 (±0.21)	-7.3% (±4.3%), ns	+0.0% (±4.7%), ns	+84.5% (±25.2%), ns	**+0.27 (±0.03), p<0.05**	**+3.76 (±0.26), p<0.05**	**+1.31 (±0.13), p<0.05**
Red clover						**Pooled:**	+0.02 (±0.02), ns	**-1.45 (±0.13), p<0.05**	**-0.43 (±0.04), p<0.05**
Mix 4	40.2 (±7.4)	1.37 (±0.25)	0.2 (±0.04)	**+76.7% (±18.5%), p=0.13**	**+74.8% (±18.5%), p=0.14**	-23.7% (±11.9%), ns	-0.06 (±0.05), ns	**-1.99 (±0.18), p<0.05**	**-0.58 (±0.05), p<0.05**
Mix 10	60.0 (±7.5)	2.10 (±0.26)	0.5 (±0.10)	**+101.0% (±21.0%), p=0.10**	**+104.2% (±20.4%), p=0.08**	+38.7% (±27.2%), ns	+0.10 (±0.04), ns	-0.91 (±0.28), ns	-0.28 (±0.08), ns
Mea. fescue Mix 10 only	78.0 (±8.5)	1.80 (±0.20)	1.6 (±0.13)	-9.3% (±9.0%), ns	-2.4% (±9.8%), ns	+53.6% (±15.0%), ns	**+0.16 (±0.02), p<0.05**	**+2.57 (±0.47), p=0.07**	**+1.01 (±0.20), p=0.09**
Bluegrass Mix 10 only	2.3 (±0.7)	0.07 (±0.02)	0.0 (±0.01)	-2.1% (±8.0%), ns	+13.1% (±12.6%), ns	**-57.8% (±10.0%), p=0.08**	**+0.42 (±0.05), p<0.05**	**-3.09 (±0.37), p<0.05**	**-1.25 (±0.12), p<0.05**
White clover Mix 10 only	1.9 (±0.5)	0.08 (±0.02)	0.0 (±0.00)	+311.3% (±90.8%), ns	+332.1% (±98.1%), ns	+10.8% (±17.3%), ns	+0.13 (±0.04), ns	**-1.29 (±0.20), p=0.07**	**-0.34 (±0.05), p=0.07**



^{15}N recovered, pct of remaining; (due to overyielding)				
	Mix 4		**Mix 10**	
H1	**6 %**	(1 %)	**6 %**	(0 %)
H2	**31 %**	(3 %)	**20 %**	(-4 %)
H3	**26 %**	(7 %)	**24 %**	(6 %)
H4	**6 %**	(0 %)	**10 %**	(2 %)

Figure S 1: Overyielding (Eqn. 9) in mixtures in four consecutive harvests (H1 to H4) of: **DM yield** (g DM m^{-2}), **N yield** (g N m^{-2}), and total **^{15}N herbage uptake** (mg ^{15}N m^{-2}). Mean values of 4 replicates (±SE). Percent change and significance of one-sided t-test for positive overyielding is shown above bars. NS: p>0.15. Bromegrass is included assuming no diversity effect, i.e. *expected values = realized values*. Excludes weeds.

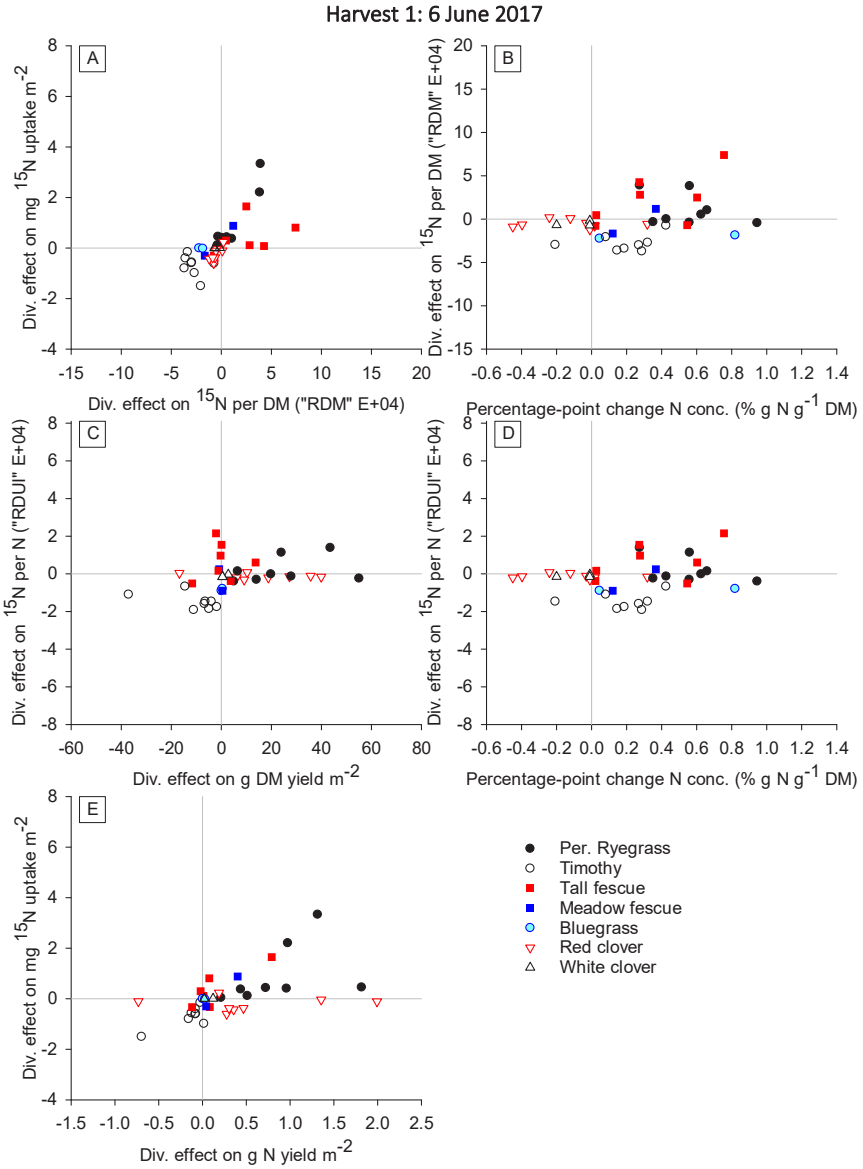


Figure S 2 (H1): Relationships between selected diversity effects, first spring harvest after ^{15}N labeling, 6 June 2017. (*Realized - expected* values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (Eqn. 6, 8; mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (Eqn. 7, 8; mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All replicates are plotted from both mixtures within this harvest.

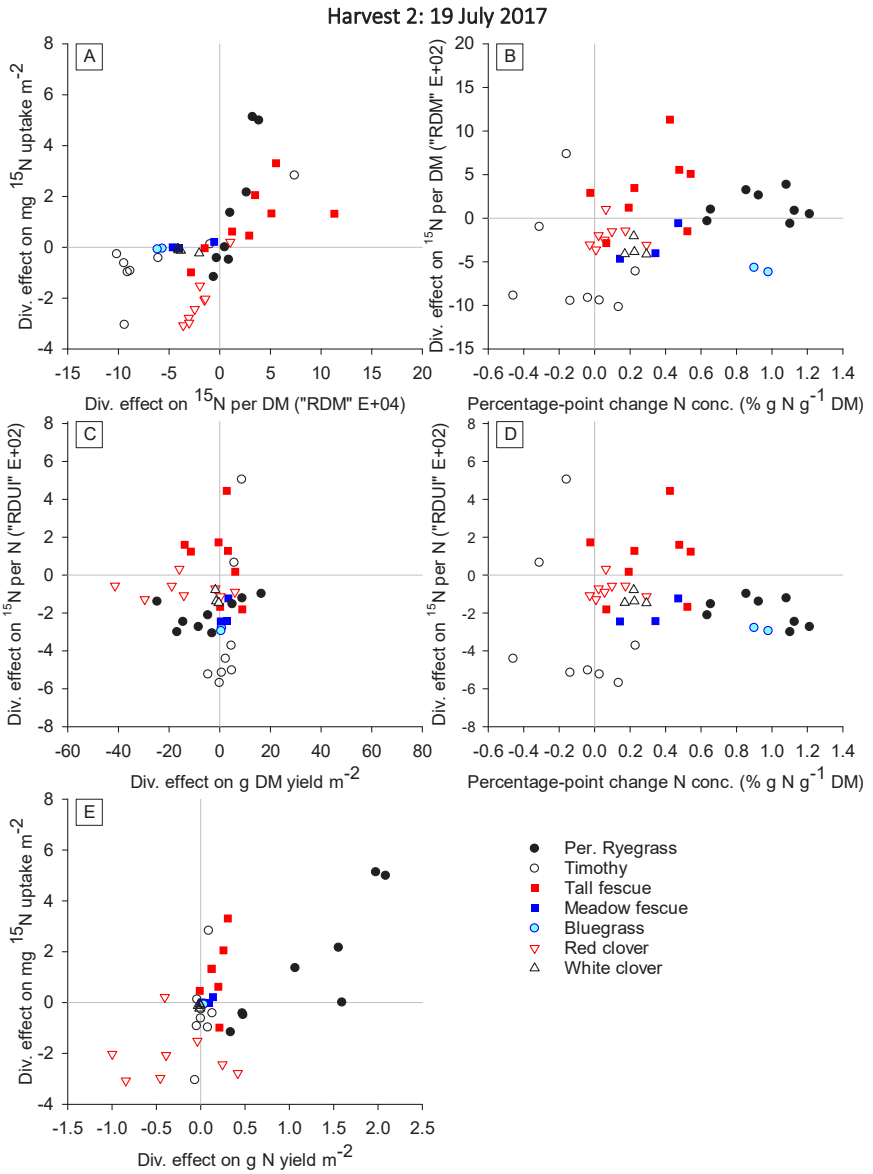


Figure S 2 (H2): Relationships between selected diversity effects, summer harvest after ^{15}N labeling, 19 July 2017. (*Realized - expected* values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (Eqn. 6, 8; mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (Eqn. 7, 8; mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All replicates are plotted from both mixtures within this harvest.

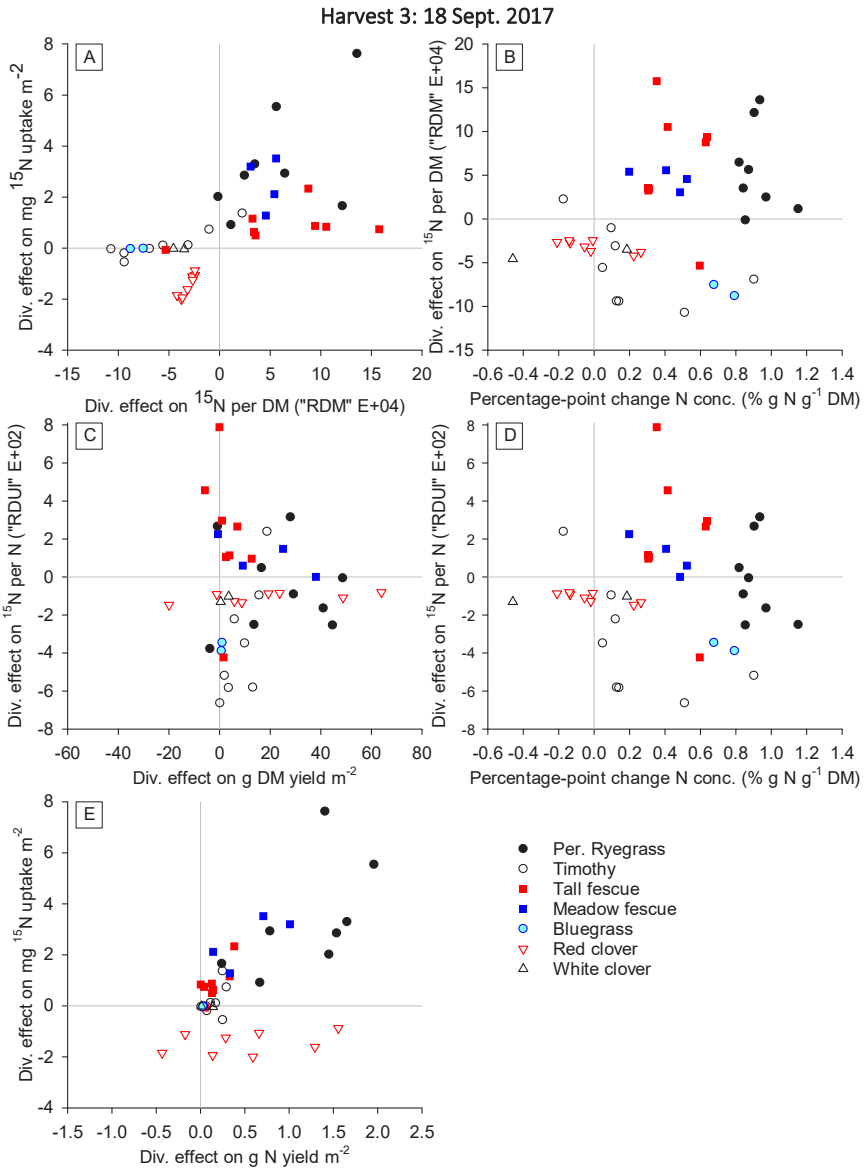


Figure S 2 (H3): Relationships between selected diversity effects, autumn harvest after ^{15}N labeling, 18 Sept. 2017. (*Realized - expected* values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (Eqn. 6, 8; mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (Eqn. 7, 8; mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All replicates are plotted from both mixtures within this harvest.

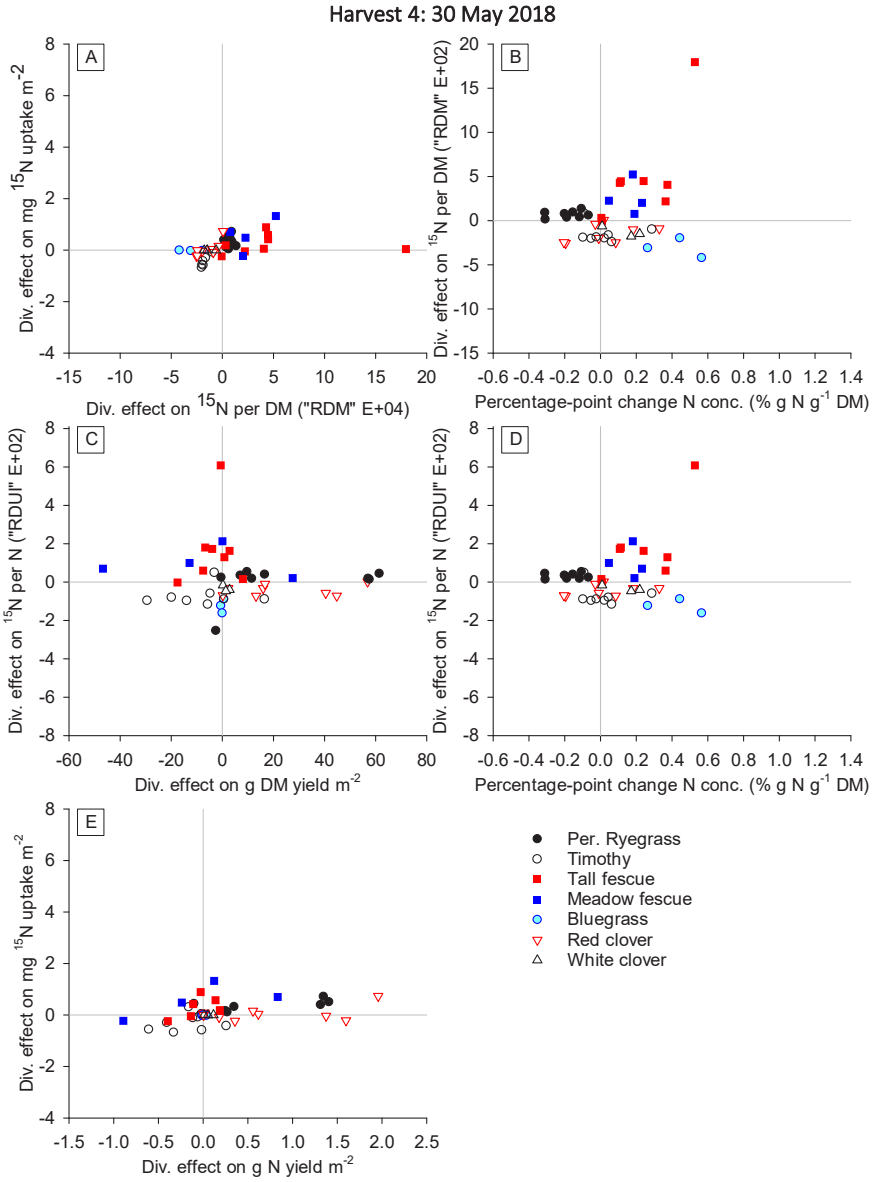


Figure S 2 (H4): Relationships between selected diversity effects, second spring harvest after ^{15}N labeling, 30 May 2018. (*Realized - expected* values, Δ for brevity) on **A:** Δ mg ^{15}N herbage uptake m^{-2} versus ΔRDM (Eqn. 6, 8; mg ^{15}N g^{-1} DM); **B:** ΔRDM versus Δ N concentration in DM (percentage-point change of g N g^{-1} DM); **C:** ΔRDUI (Eqn. 7, 8; mg ^{15}N g^{-1} N) versus Δ g DM yield m^{-2}; **D:** ΔRDUI versus Δ N concentration in DM; **E:** Δ mg ^{15}N herbage uptake m^{-2} versus Δ g N yield m^{-2}. All replicates are plotted from both mixtures within this harvest.

Paper III

ENVIRONMENTAL RESEARCH
COMMUNICATIONS

PAPER • OPEN ACCESS

Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH

To cite this article: Erin Byers *et al* 2021 *Environ. Res. Commun.* **3** 015001

Environmental Research Communications

PAPER

Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH

Erin Byers, Marina A Bleken and Peter Dörsch

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, PO Box 5003, 1432 Ås, Norway

E-mail: erin.byers@nmbu.no

OPEN ACCESS

RECEIVED
9 August 2020

REVISED
18 December 2020

ACCEPTED FOR PUBLICATION
22 December 2020

PUBLISHED
19 January 2021

Keywords: managed perennial grasslands, off-season nitrification, off-season denitrification, clovers, diversity effects, liming

Supplementary material for this article is available online

Abstract

Inclusion of legume species into grass leys reduces nitrogen (N) fertilizer need but increases the risk of freeze-thaw induced N_2O emissions. We investigated how liming and presence of clover affect N_2O accumulation under snowpack and its emission during freeze-thaw cycles in autumn and spring under sub-boreal conditions. A field experiment was performed in southern Norway in limed and control plots containing grasses only (fertilized with 270 kg N ha^{-1} yr^{-1}), a grass-red clover mixture (fertilized with 140 kg N ha^{-1} yr^{-1}) and unfertilized pure red clover. Soil air samples were collected at 8, 24, and 40 cm depths and analyzed for gas concentrations including N_2O, and N_2O fluxes measured by a fast-chamber robot. Red clover produced more N_2O than the grass-only plots during freeze-thaw cycles in autumn and spring and accumulated more N_2O under snow cover (emissions were not measured during this period). Contrary to expectations, limed red clover plots emitted more N_2O than control plots during freeze-thaw cycles. Liming reduced subnivean N_2O accumulation in grass-only but not in grass-clover or pure clover plots. After spring fertilization, grass-only plots had larger N_2O emissions than red clover plots. Our data suggest that winter-sensitive, N-rich clover biomass fuels decomposition and nitrification, thereby increasing NO_3^- and depleting O_2, resulting in increased N_2O emissions from denitrification. Although liming of pure clover leys exacerbated the risk of high N_2O emissions during freeze-thaw, this effect was not observed in grass-clover mixtures. Interestingly, grass-clover mixtures also emitted less N_2O than expected from their proportions and the emissions recorded in pure grass and clover stands. This warrants further studies into off-season functional diversity effects on N cycling and N_2O loss in temperate and boreal forage production.

1. Introduction

Human activity has doubled the amount of nitrogen (N) transferred annually from the atmosphere to terrestrial biomass pools, primarily through synthetic fertilizer application and increased use of legumes in agriculture (Vitousek *et al* 1997, Fowler *et al* 2013). Nitrous oxide (N_2O) emissions from the biosphere to the atmosphere have nearly doubled since preindustrial times, with agriculture accounting for most of this increase (Ussiri and Lal 2013, Tian *et al* 2019). In 2010, N_2O contributed an estimated 6.2% of the annual greenhouse gas emission (Gt CO_2-eq yr^{-1}) to the atmosphere (IPCC 2014), where it also contributes to ozone depletion (Ravishankara *et al* 2009). A warmer and wetter climate is predicted to increase the magnitude and variability of N_2O emissions from agriculture (Griffis *et al* 2017). At the same time, agricultural soils in sub-boreal Europe have shown larger peak fluxes and larger variability in annual N_2O emissions than in temperate oceanic Europe, which may in part be due to large episodic winter emissions triggered by freeze-thaw in addition to growing-season emissions triggered by fertilizer additon and rainfalls (Freibauer and Kaltschmitt 2003). Croplands in climates experiencing freeze-thaw cycles can emit more than half of their annual N_2O during winter and early spring (Christensen and Tiedje 1990, Flessa *et al* 1995, Kaiser *et al* 1998, Wagner-Riddle *et al* 2017) despite reduced decomposition due to low temperature.

As in wetting-drying cycles, the primary pathway for N_2O triggered by freeze-thaw is thought to be heterotrophic denitrification (Kim *et al* 2012, Risk *et al* 2013, Congreves *et al* 2018). Nitrification, which can also produce N_2O, albeit at a smaller mole fraction (Mørkved *et al* 2007), is rarely discussed as a limiting step in overwinter N_2O production, perhaps because NO_3^- existing at any time in winter is sufficient to explain observed N_2O emissions by denitrification. Yet, before denitrification can occur, NO_3^- (and NO_2^-) must be made available by oxidation of NH_4^+, mineralized either from decomposing organic material or fertilizer. Recent findings have identified ammonia-oxidizing archaea (AOA) playing an important role for NH_4^+ oxidation in soils subjected to freezing and thawing (Tzanakakis *et al* 2020).

While agronomic practice often seeks to minimize residual mineral N at the end of the growing season, freeze-thaw cycles release labile N and C from crop residues and soil organic material. Labile N fuels nitrification, and labile C fuels respiration, including denitrification. Off-season N_2O emissions have been attributed to labile substrates released from frost-killed biomass (plants and microbes) or protected soil organic matter, rather than to mineral N (N_{min}) status prior to freezing (Christensen and Christensen 1991, Müller *et al* 2002, Mørkved *et al* 2006, Russenes *et al* 2019). Denitrification can continue at temperatures several degrees centigrade below zero given an adequate amount of unfrozen water (Teepe *et al* 2001, Öquist *et al* 2004, Monson *et al* 2006) and adequate availability of carbon sources (Sehy *et al* 2004).

In a warming climate, regions with pronounced winters may experience delayed or absent snowpack, exposing soil to more frequent freeze-thaw cycles (Groffman *et al* 2001). Every freeze-thaw cycle has potential to create conditions conducive to N_2O formation, with early and strong freezes releasing more substrate than subsequent or weaker freezes as frost-sensitive organic matter becomes depleted (Priemé and Christensen 2001, Koponen and Martikainen 2004). Snow has an insulating effect which can decrease freeze-thaw intensity and thus lessen N_2O-producing events (Maljanen *et al* 2007, Ruan and Robertson 2017). Nitrous oxide produced under snowpack may equilibrate with the atmosphere by diffusing through the snowpack (Sommerfeld *et al* 1993, Graham and Risk 2018) or be trapped below frozen soil or surface ice layers (Burton and Beauchamp 1994). Under conditions of reduced atmospheric exchange, initial nitrification fueled by freeze-thaw driven substrate release may contribute to subsequent denitrification by consuming available oxygen and inducing coupled nitrification-denitrification (Kremen *et al* 2005). Subnivean N_2O can then accumulate over winter and be released at spring thaw, followed by (Risk *et al* 2014) *de novo* N_2O production (Röver *et al* 1998, Teepe *et al* 2001, Russenes *et al* 2019). The relative contribution of these two mechanisms to spring N_2O emissions seems to vary and is difficult to quantify based on soil air concentrations and flux measurements alone (Risk *et al* 2013, 2014).

Combining legumes with grasses in multi-species stands can improve nitrogen use efficiency (NUE) by stimulating N fixation in legumes and transferring symbiotic and non-symbiotic N to grasses, leading to N yields similar to those of pure legume stands (Nyfeler *et al* 2011). However, inclusion of clovers can cause N_2O production—not from the process of biological nitrogen fixation (BNF) itself, but due to decomposition of biomass with a low C:N ratio (Rochette and Janzen 2005, Carter and Ambus 2006). Inclusion of legumes has also been shown to increase NO_3^- leaching in grassland mixtures (Leimer *et al* 2015) and cover crops (Gabriel *et al* 2016). The N_2O tradeoff of replacing fertilizer with legumes depends on the level of fertilization of the system, and conditions influencing decomposition of leguminous biomass. Models by Fuchs *et al* (2020) based on experiments in mostly temperate locations in Europe indicated that replacing fertilizer with legumes reduced N_2O while maintaining productivity. However, boreal grasslands which undergo freeze-thaw risk large off-season N_2O emissions from legumes, likely negating any N_2O reduction from using less fertilizer (Virkajärvi *et al* 2010). The importance of off-season N_2O emissions in legume-containing cover crops was also seen in a meta-study by Basche *et al* (2014), showing that legume-containing cover crops do not improve the N_2O footprint of the cropping system into which they are incorporated when accounting for both the growing and off seasons.

In perennial systems, winter N_2O dynamics may be closely tied to overwinter survival of the plants. During winter, much of the biomass is located above-ground at the soil surface as stubble and below-ground in root systems, both of which may be enriched with N translocated to storage organs late in the season (e.g. Garten Jr *et al* 2010). In sub-boreal climates, winter survival of grassland species is generally poor, especially of non-native species, due to poor triggering of overwinter survival mechanisms, although breeding efforts may improve this (Østrem *et al* 2015). Legumes are less winter-hardy than grasses, increasing the risk for freeze-thaw induced N loss (Woledge *et al* 1990, Sturite *et al* 2007a, 2007b). Information remains sparse on the freeze-thaw driven effect of clovers on N_2O production in grasslands.

Liming has been proposed as a way to mitigate denitrification-derived N_2O emissions, because low pH prevents maturation of a functioning N_2O reductase in known denitrifiers (Bakken *et al* 2012, Liu *et al* 2014). If denitrification is the predominant source for freeze-thaw induced N_2O emissions, raising the pH of acidic soil by liming should improve the ability of denitrifiers to reduce N_2O to N_2 and potentially lower N_2O emissions. Russenes *et al* (2016) demonstrated a significant effect of natural small-scale pH variability on off-season N_2O

emissions in SE Norway in a wheat stubble field, but to the best of our knowledge, there are no studies so far testing the effect of liming on off-season N_2O turnover in perennial grasslands *in situ*.

To achieve a detailed account of N_2O turnover under variable conditions throughout a sub-boreal winter, and to explore whether raising soil pH by liming could be an effective strategy to mitigate off-season N_2O emissions in clover-rich leys, we established a field study in southeastern Norway monitoring both belowground and emitted N_2O, as well as soil moisture and temperature. Swards containing only grasses, red clover in pure stand, or a grass-red clover mixture were used, each with two pH levels. The experiment ran from late autumn throughout winter and spring thaw.

The following hypotheses were tested: (1) As clover residues release more N-rich substrates upon freeze-thaw than grass residues, overwinter accumulation of N_2O in soil and subsequent emission will be largest in pure red clover stands, smallest in grass-only stands, and intermediate in the red clover - grass mixture. (2) Subnivean N_2O accumulation and subsequent emission will be smaller at higher soil pH because of more complete denitrification.

2. Methods

2.1. Study site

We used an existing plot experiment located on the NMBU research farm in Ås, Southern Norway, approx. 20 km south of Oslo (59°39′47‘N, 10°45′42‘E). The soil is classified as an Umbric Epistagnic Retisol (IUSS 2015), and is artificially drained at about 1 m depth. The top 20 cm of soil contains 2.9% organic carbon and 0.26% organic N (C:N ratio 11.1). The soil texture is 27% clay, 48% silt and 25% sand with bulk density (BD) of 1.18 g cm^3 at 10–15 cm depth, 1.53 g cm^3 at 25–30 cm and 1.65 g cm^3 at 40–45 cm (Bleken *et al* unpublished; table S.1 (available online at stacks.iop.org/ERC/3/015001/mmedia)).

In September 2014, before sowing the leys, a liming experiment was established by applying 23 t ha^{-1} dolomite to the surface in two stages; half of the dolomite was incorporated to 20 cm soil depth by ploughing, followed by harrowing the other half to 10 cm depth, resulting in a pH contrast between unlimed control (pH 5.18) and dolomite treated plots (pH 6.09). Different grassland swards were sown into limed and unlimed plots in May 2015 according to seeding rates shown in table S.2: grass-only swards containing timothy (*Phleum pratense* L. cv. Grindstad), perennial ryegrass (*Lolium perenne* L. cv. Figgjo), meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv. cv. Fure), tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort. cv. Swaj), and common meadow grass (*Poa pratensis* L. cv. Knut); a mixture combining the aforementioned grasses with red clover (*Trifolium Pratense* L. cv. Lea); and pure red clover swards. The over-winter study took place in the third production year (2017–2018). The six treatment combinations of dolomite-limed and control plots in grass-only (G-dol and G-con), grass-clover mixture (M-dol and M-con) and pure red clover (R-dol and R-con) were replicated 4 times and fully randomized (figure S.1).

Fields were harvested for silage production three times per growing season, and fertilization was split into three applications: the largest dose (45%) in early spring, and the remainder following the first and second harvest. Grass-only plots received in total 270 kg N ha^{-1} yr^{-1}, grass-red clover mixture plots 140 kg N ha^{-1} yr^{-1} and pure red clover plots did not receive any fertilizer. Prior to the winter experiment, the field was fertilized on August 1, 2017, and was harvested Sep 25, 2017, with little regrowth after the last harvest. Spring fertilization took place on April 30, 2018.

The nearest weather station at NMBU, Ås (59°39’37.8‘N, 10°46’54.5‘E), recorded an average yearly temperature of 5.7°C and precipitation of 795 mm from 1971–2000 (Wolff *et al* 2018, 2019). Nine of the 24 experimental plots were in an area of the field shaded most of the day during winter by a tree line approximately 100 m to the south, blocking the Sun which has a low elevation angle in winter months (figure S.1). This area included five of the eight grass-only plots.

2.2. Yields, pH and early winter mineral N

On September 25, 2017 a 6.2 by 1.5 m swath was harvested from each plot and fresh weight yields recorded using a Haldrup F-55 grass harvester (J. Haldrup a/s, Denmark). Biomass subsamples from each plot were collected, mixtures were botanized into clovers and grasses, and all subsamples were weighed before and after being dried at 60°C to calculate dry matter (DM) yields per m^2.

Soil samples were taken on December 8, 2017 from 0–10 cm, using four 16 mm diameter soil cores per plot. To avoid disturbance of the gas measurements, the samples were taken roughly one half-meter away from the probes used for soil air sampling and the area measured for surface flux (see Methods 2.3, 2.4). Soil samples were sieved and frozen on the day of collection and later analyzed for 1 M KCl-extractable $NO_2^- + NO_3^-$ using the Griess reaction with Vanadium (III) chloride (Doane and Horwáth 2003), analyzed for NH_4^+ using the Berthelot

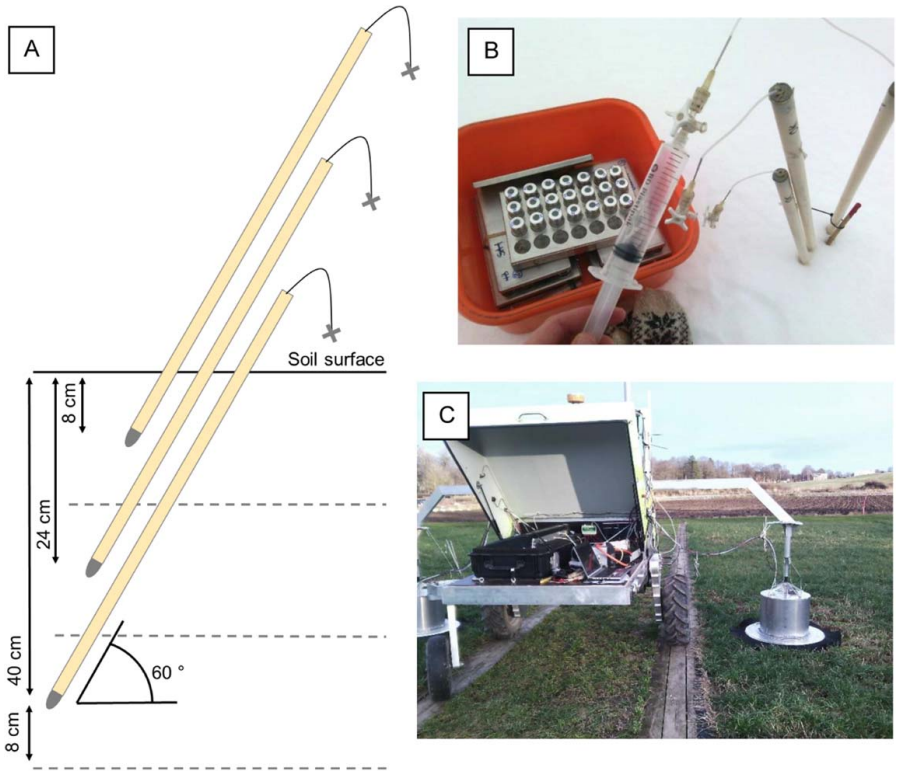


Figure 1. (A) Schematic of soil air probes and setup (See Methods, 2.4 Soil air). Marked depths show approximate placement of porous cups. Samples collected from each depth represent the center of the soil volume demarked by dashed lines, which are halfway between each sampling depth. (B) Photograph of collecting soil air samples from a sampling station in snow cover. (C) Photograph of autonomous field flux robot (FFR). Figures and photographs by Erin Byers.

reaction with sodium salicylate, sodium nitroprusside, and sodium dichloroisocyanurate (Krom 1980), and analyzed for pH in a 1:2.5 slurry with 10 mM $CaCl_2$.

2.3. Soil air

Soil air samples were taken approximately weekly from November 8, 2017 to April 28, 2010 by soil air probes permanently installed at 8, 24 and 40 cm depth (figures 1(A), (B); sampling dates in table S.3). Because soil air probes had to be removed before spring fertilization, we did not take soil air samples in May. The probes are described in detail by Nadeem *et al* (2012). Briefly, they consist of an air-permeable cup (pore diameter 100 μm) glued to a 3.3 cm outer diameter PVC tube, through which a 0.97 mm inner diameter PTFE tube runs to connect the void of the porous cup with a 3-way valve above the soil surface. The samplers were installed in the first week of November 2017 into pre-augered holes at a 60° angle to the soil surface in order to minimize preferential water flow along the tubes. At each sampling, 10–15 ml were withdrawn using a 20 ml plastic syringe and injected into 10 ml He-washed and evacuated rubber septa-capped glass vials. On occasions of high VWC, water entered the lines and we were unable to obtain soil air samples. Moisture in the PTFE tubes sometimes froze, rendering a sampling location unusable for extended periods.

Soil air samples were analyzed for CO_2, N_2O, CH_4, N_2 and O_2 mixing ratios by gas chromatography (GC). The GC system (7890A, Agilent Technologies, California, USA) is described in detail by Nadeem *et al* (2015). An autosampler connected via a peristaltic pump (222 XL and MINIPULS 3, both from Gilson, Wisconsin, USA) conveys approximately 1 ml from the septa-capped vials to the GC, which is equipped with a 30 m wide-bore Poraplot Q (0.53 mm) column to separate N_2O, CO_2, CH_4 from bulk air, and a 60 m wide-bore 5 A molesieve column to separate N_2, O_2 and Ar. Helium was used as a carrier gas. An inline thermal conductivity detector (TCD) quantifies O_2 and N_2 and high concentrations of N_2O and CH_4, an electron capture detector (ECD) N_2O concentrations at ambient levels, and a flame ionization detector (FID) quantifies CH_4.

2.4. Surface fluxes

N_2O emissions were estimated in all plots using an automated fast-box technique (Hensen *et al* 2006, Cowan *et al* 2014) attached to a mobile autonomous field flux robot (figure 1(C)). The robot was programmed to move on boardwalks between the plots (figure S.1). This allowed for frequent measurements in the period of November 14 to December 12, when soils were exposed to freeze-thaw, during spring thaw from April 6 to 27, and after spring fertilization from May 2 to 11 (table S.3). Robot operation was not possible between January 12 and April 6 due to a continuous deep snowpack.

The robot mechanics and navigation were designed by Adigo AS, Norway, and the gas measurement system and software by Lars Molstad and Jan Reent Köster at the Norwegian University of Life Sciences (NMBU). The robot lowers two collarless chambers lined at the bottom with cellular rubber and windbreak skirting onto the field surface, and circulates air from one chamber at a time through a Tunable Diode Laser N_2O/CO analyzer (DLT-100, Los Gatos Research, California, USA) and a CO_2/H_2O infrared gas analyzer (LI-840A, LI-COR Biosciences, Nebraska, USA). One or both chambers can be measured while stationed at a single waypoint: when target plots lie on both sides of the robot and both chamber can be used, the flow is switched by help of a multiplexer every 20 s between the two chambers and the analyzers, ignoring 6 s of data during the transition, effectively giving 14 s long continuous readings (1 Hz sampling frequency) every 40 s over the three-minute chamber deployment time. Flux rates of N_2O were estimated from the slope of the concentration versus time over 120 s. To check chamber tightness, we also inspected CO_2 measurements taken during the same time period, assuming that in the absence of leakage, CO_2 concentrations should increase linearly.

2.5. Soil temperature and moisture

In order to continuously measure soil temperature and soil volumetric water content (VWC), we installed dataloggers (Decagon Em50) at four locations within the field, including one in the shaded area (figure S.1), each connected to five combined time-domain reflectometry (TDR)—thermistor probes (5TM VWC + Temp, Decagon Devices, Inc., Washington, USA). At each location, probes were placed at 5, 24, and 40 cm depth (two probes per logger at 5 cm depth), as well as within the plant stubble just under the soil surface.

2.6. Frost tubes

To monitor freezing depth, frost tubes were installed at two locations within the field, one in the Sunny and one in the shaded area (figure S.1). The frost tubes were constructed and filled with 0.05 percent methyl blue solution according to McCool and Molnau (1984). Freezing depth was recorded each time soil air samples were taken.

2.7. Calculations

2.7.1. Accumulation of N_2O in soil

Of all soil air measurements, 24.8% were discarded due to liquid water or ice blocking the tubing. For the purpose of integrating N_2O accumulation over time, missing values (ppm N_2O) were interpolated from values at other depths in the same replicate. In 4% of these, none of the three depths were measurable and values were interpolated from previous and subsequent observations from the same replicate. 12% were interpolated from a valid measurement at only one depth, and 8% from valid measurements at two depths.

To estimate the amount of N_2O stored in the soil matrix down to 48 cm depth (g N_2O-N m^{-2} 0.48 m^{-1}) at each sampling event, we converted N_2O concentrations (ppm) to N mass assuming equilibrium between gaseous and dissolved N_2O (see figure 2 as an example). The soil volume was divided into three layers of 16 cm depth each, and the soil air probe installed in the center of each layer was taken as indicative for the gas concentrations for that layer (figure 1(A)). We determined percent air-filled pore space (AFPS) in each layer according to equation (1), using existing plot-wise bulk density data from 2014 (Bleken *et al* unpublished; table S.4).

$$AFPS = 1 - \left(\frac{VWC + VIC}{1 - \frac{BD}{2.64}} \right) \tag{1}$$

where VWC is the volumetric water content in % as measured by TDR, VIC the volumetric ice content in % estimated by equation (2) (below), BD the soil bulk density in g cm^{-3} and 2.64 g cm^{-3} the assumed soil particle density.

We assumed ice crystals in the soil to freeze out all gases; that is, frozen volume would not contain N_2O and must be excluded from the calculated AFPS. Upon soil freezing, VWC as measured by TDR dropped sharply because the probes do not detect ice. Without correction, this undetected ice volume would be erroneously considered part of the AFPS. The excluded volumetric ice content (VIC) was defined as:

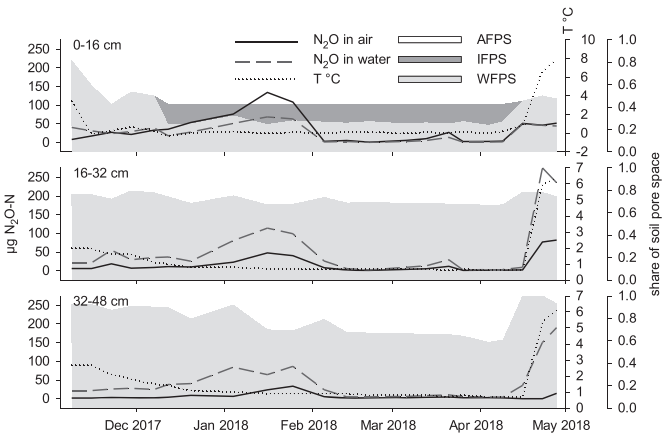


Figure 2. Example of N_2O partitioning to gaseous and dissolved fractions in different soil layers. Shaded areas depict the shares of soil pore space occupied by air (AFPS), water (WFPS), and ice (IFPS). Lines are estimated gaseous and dissolved amounts of N_2O in μg N 0.16 m^{-3} present in in each layer, as well as soil temperature measured by the nearest TDR-thermistor probe. Data: G-dol plot #112.

$$VIC = VWC_{t0} - VWC_t \quad (2)$$

where VWC_{t0} is the VWC at the last measured temperature before freezing, and VWC_t the VWC measured by TDR for measurements between freezing until WC_t again equals VWC_{t0} (figure 2).

For simplification, we did not consider increased volume of frozen water due to expansion, or the phenomenon that moisture may be drawn by convection towards the freezing front.

We assumed that gases dissolved in soil water were at equilibrium with gases in soil air at the time of sampling and calculated N_2O amounts in both soil air and soil water using temperature corrected mole volumes and Henry's Law with the Van't Hoff correction for temperature (Sander 2015).

For comparing N_2O accumulation in the soil of different treatments, we used a time integral of the mass of N_2O-N over the duration of its presence. This was necessary because during prolonged periods of impeded soil-air exchange, maximum N_2O concentrations in the replicate plots were reached on different dates, and some plots subsequently decreased in N_2O concentration long before others, indicating possible release or subnivean N_2O consumption. The resulting integral (g N_2O-N m^{-2} 0.48 m^{-1} * days) thus represents the total amount of gaseous and dissolved N_2O-N present belowground during a given period in days.

2.7.2. Cumulative N_2O surface fluxes

We estimated cumulative N_2O emissions plot-wise by linear interpolation between instantaneous flux rates measured at each sampling. On about a quarter of sampling dates in the spring, we had the opportunity to measure multiple times throughout a single day, more than one hour apart (see table S.3). For these days we interpolated between each measurement rather than averaging measurements.

Of 1104 fluxes estimated, 24 measurements (2%) taken on the same day were excluded because the recorded N_2O concentration right after chamber deployment was above ambient (up to 0.8 ppm). This occurred on a day with snow cover, suggesting that deploying the chambers released N_2O stored in the snowpack, which subsequently re-equilibrated, giving unrealistically high negative fluxes (up to -700 μg N_2O-N m^{-2} h^{-1}). 122 measurements (11%) were included but set to zero flux because the trend in N_2O concentration over time in the flux chamber was not significantly different from zero ($p>0.05$) and thus beyond the detection limit of the method ($\sim$5 μg N m^2 h^{-1}). 234 (21%) of the estimated N_2O fluxes were negative (indicating N_2O uptake by the soil, on average -12 μg N m^2 h^{-1} and at most -62 μg N m^2 h^{-1}). These measurements had reasonable starting values (0.325 ppm $\pm$ 0.15 ppm N_2O) and p-values below 0.05, and were thus included in the study. We did not exclude any N_2O measurements based on CO_2 data. Inspection of CO_2 measurements showed linear concentration changes, indicating the chambers had even contact with the surface. Some measurements atop snow and ice showed no increase in CO_2. On two days (January 9 and May 11) the CO_2 analyzer was malfunctioning and did not record measurements, although the N_2O analyzer was functioning normally.

2.7.3. Proportionality of fluxes to clover share in mixtures

To explore plant diversity effects on N_2O emissions, we calculated expected N_2O fluxes in mixture plots during each time period based on the share of clover in mixture DM yield and the average flux per DM yield in the

Table 1. (A) Dry matter yields (g m^{-2}) and share of clover in dry matter of aboveground biomass harvested September 25, 2017; (B) pH_{CaCl2} and (C) extractable mineral N (mg kg DW $soil^{-1}$) sampled on December 8, 2017 from 0–10 cm depth. Mean (±SE) of 4 replicates except where noted. Letters indicate Tukey groupings ($p<0.05$).

	A		B		
	DM Yield g m^{-2}	Clover share	pH_{CaCl2}		
G-con	570.2 (±40.2) A	0	5.17 (±0.23) B		
G-dol	567.2 (±21.7) A	0	6.04 (±0.09) A		
M-con	510.3 (±31.5) AB	0.49 (±0.02) A	5.14 (±0.03) B		
M-dol	498.0 (±30.3) AB	0.38 (±0.06) A	6.06 (±0.16) A		
R-con	340.8 (±17.9) C	1	5.23 (±0.09) B		
R-dol	389.5 (±35.3) BC	1	6.17 (±0.07) A		
	C				
	mg NH_4^+ N kg^{-1}	mg NO_3 N kg^{-1}	Total N-min	Total N-min, 2 outliers removed	
G-con	1.27 (±0.28)	0.21 (±0.18)	1.48 (±0.14) A	1.48 (±0.14)	D
G-dol	2.83 (±1.98)	1.37 (±2.26)	4.20 (±2.12) A	2.08 (±0.18, n=3)	CD
M-con	2.35 (±0.36)	1.47 (±0.33)	3.82 (±0.09) A	3.82 (±0.09)	BC
M-dol	5.45 (±4.85)	3.45 (±2.95)	8.90 (±3.86) A	5.06 (±0.47, n=3)	B
R-con	1.79 (±0.19)	2.50 (±1.15)	4.29 (±0.61) A	4.29 (±0.61)	B
R-dol	3.00 (±1.06)	4.15 (±0.63)	7.15 (±0.49) A	7.15 (±0.49)	A

grass-only and pure red clover stands (equation (3)). Expected fluxes in limed and control mixture plots were calculated separately.

For each mixture plot *i* in pH treatment *p:*

$$Flux_{MixExpected,\,ip} = DM_{ip}\left[CSHARE_i\left(Avg\left(\frac{Flux}{DM}\right)_{Clover,p}\right) + (1 - CSHARE_i)\left(Avg\left(\frac{Flux}{DM}\right)_{Grass,p}\right)\right] \quad (3)$$

where CSHARE is the proportion of clover in each mixture plot by DM yield.

2.8. Statistics

Cumulative N_2O fluxes and time-integrated N_2O accumulation in the soil were analyzed both for the entire duration of the experiment and for selected periods representing different weather and soil physical conditions throughout the experiment.

The effects of pH, species and their interaction on each response variable (cumulative N_2O fluxes and time-integrated N_2O accumulation) were tested with an ANOVA factorial model using the *anova* function in the R software package, version 3.6.1. After testing for normality, it was found necessary to transform the response variables to their natural logarithms. *Post hoc* Tukey tests were applied to identify differences between species, using *simple.glht* from the *mixlm* package for R, $\alpha = 0.05$. For each time period, pairwise comparisons of the natural log of each species' response variable (ignoring pH) indicated where species showed different effects. In selected time periods, the pH effect on the log of the response variable was tested independently for grass-only swards, since including the other swards strongly increased the MSE, and thus reduced the power of the analysis on the pH effect.

3. Results

3.1. Yields, pH and mineral N status of the soil

At the last harvest before the experiment, grass-only plots (limed, G-dol and control, G-con) yielded around 570 g DM m^{-2}, significantly more than pure red clover plots (365 g DM m^{-2}), with the mixtures yielding in between (table 1(A)). Liming had no significant effect on yields irrespective of plant species, although limed red clover plots (R-dol) tended to yield more (390 g m^{-2}) than red clover control plots (R-con; 341 g m^{-2}). The experiment took place three growing seasons after sowing of the swards, and grass-clover mixture plots contained more red clover than the initial 10% seeding weight (table 1(A)). Limed mixture plots (M-dol) tended to contain less red clover by percent of DM (38%) than mixture control plots (M-con; 49%), although this difference was not significant.

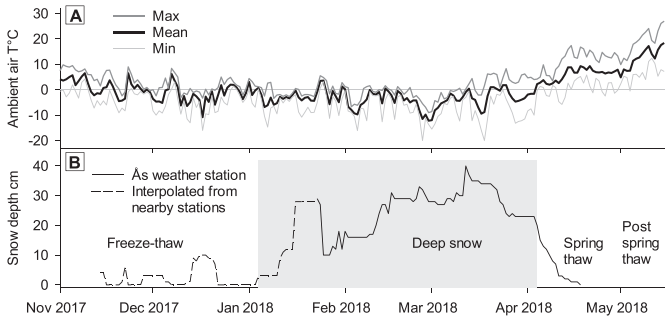


Figure 3. Weather conditions from November 1, 2017 to May 15, 2018 (59°39'37.8'N, 10°46'54.5'E; Wolff *et al.* 2018, 2019). (A) daily mean, maximum, and minimum temperature °C. (B) daily recorded snow depth, cm. The dashed line indicates snow depth interpolated from notes and two nearby weather stations. The shaded box designates the period of deep snow cover.

Soil samples were taken on December 8, 2017 at 0–10 cm depth. The soil pH_{CaCl2} was 5.18 (SE 0.075) in control plots, and significantly higher, 6.09 (SE 0.060), in plots limed with dolomite (table 1(B)). The difference in pH between control and limed plots was significant in all sward types. The soil N_{min} content was generally low, 1 to 8.5 mg N kg^{-1} dry soil (with no detectable nitrite; table 1(C)). There were two outliers with high N_{min} values adjacent to each other in the shaded area of the field, which had among the highest BD and C content in the topsoil relative to other plots (G-dol plot 628 and M-dol plot 527; table S.4). Excluding these, the pure red clover plots had significantly higher N_{min} contents than grass-only plots (Tukey tests, $p < 0.05$). Limed plots tended to have higher N_{min} values on December 8 than the control plots, particularly in pure red clover.

3.2. Weather and soil physical conditions

We measured surface fluxes and N_2O accumulation in the soil from late fall 2017 into spring 2018. Four periods were identified based on weather and soil conditions (figures 3; 4(E), (F)): (I) 'Freeze-thaw,' throughout late fall and early winter, during which the soil underwent successive, partly diurnal freeze-thaw cycles of increasing intensity in absence of snow cover. (II) 'Continuous deep snow cover' from early January onwards, with an ice layer gradually forming at the soil surface below the snow from daytime snowmelt. Snowpack insulated the soil such that temperature at 5 cm depth varied little between 0.0 and 0.5 °C, dipping below the freezing point only with very cold ambient air temperatures. III) 'Spring thaw' starting on April 1 when snowmelt began, followed by thawing at 5 cm soil depth and receding of the freezing front towards the soil surface (figure S.2). By mid-April the soil was completely thawed and soil temperature at 5 cm depth fluctuated daily between 5 and 10 °C until May. IV) 'Post spring thaw' in early May, when mean ambient air and soil temperatures at 5 cm depth began to rise above 10°; during this period we took only flux measurements.

Freezing was limited to the topsoil, with temperatures as low as −1.7 °C at 5 cm depth in the shaded area of the field; temperatures stayed above 0.4 °C at 24 and 0.6 °C at 40 cm depth (figures S.3(E), (G)). Frost tubes registered a maximum freezing depth on January 16 of 12 cm in the Sunny part of the field and 15 cm in the shaded area (figures S.1; S.2).

Soil temperature and VWC measurements from the TDR-thermistor probes placed in different areas of the field were in close agreement with one another (figure S.3). Logging station #1, placed in the shaded area of the field (figure S.1), measured lower soil temperatures and VWC at 5 and 24 cm depth than the other stations and registered lower minimum temperatures just below the soil surface during freezing periods before snow cover. Soil temperatures at 40 cm depth were similar between logging stations regardless of location in the field.

Overwinter survival of pure red clover stands was poor, with bare soil patches and uneven and delayed regreening on some of the plants throughout the spring thaw. By contrast, grass-only and mixture plots had fully green ground cover soon after snowmelt.

3.3. Soil air gas concentrations and N_2O accumulation

During periods of reduced soil-atmosphere exchange due to soil freezing or snow cover, soil O_2 and CH_4 concentration decreased while concentrations of CO_2 and N_2O increased (figures 4(A)–(C); for replicate measurements at each depth, see figure S.4). The concentration measurements per depth showed some evidence that topsoil layers were the first to reach elevated N_2O concentrations after the onset of soil freezing. Likewise, during spring thaw, upper soil layers were the first to show reduced N_2O concentrations. During the winter, when diffusion between soil and atmosphere was restricted for longer periods, gas concentrations were close to

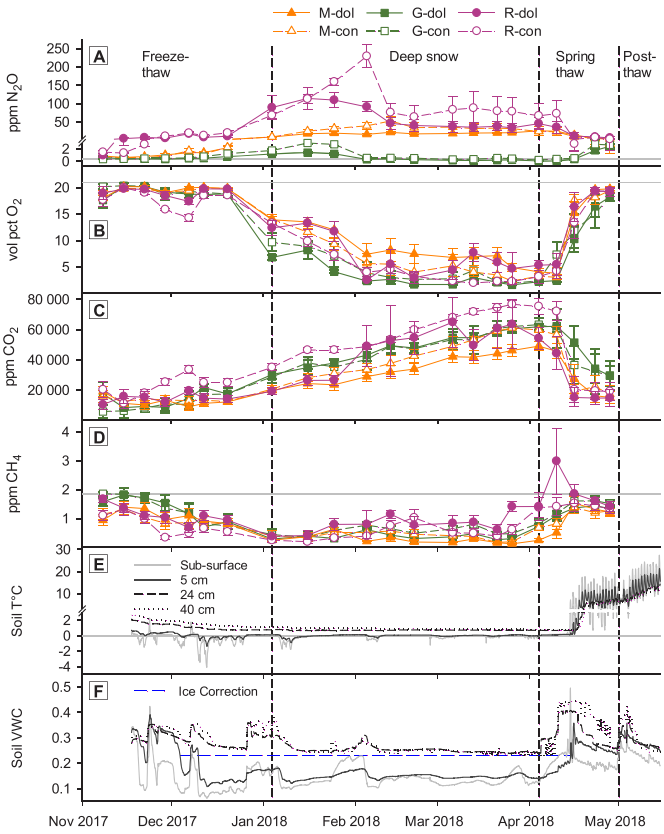


Figure 4. Concentrations of (A) N_2O, (B) O_2, (C) CO_2 and (D) CH_4 in soil air for each treatment. Mean (±SE) of 3 replicates at 3 depths (n=9). Concentration data for individual depths and plots are available in supplementary figure S.3. Gray lines indicate the ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95 vol%), and CH_4 (1.85 ppm). (E) Soil temperature °C and (F) soil VWC at each depth (mean of 4 logging stations). The blue dashed line in (F) shows the assumed soil VWC including frozen water. Vertical dashed lines indicate seasonal divisions; no soil air samples were taken post-thaw.

equal between the three layers, suggesting that diffusion in the soil AFPS was unrestricted below the freezing front. The soil VWC was greatest in the deeper layers resulting in more N_2O being dissolved in water than present in the small remaining air fraction (figure 2).

On December 7, N_2O concentration in soil air reached its first peak, following a major soil freeze as evidenced by a sudden drop in soil temperature and VWC at 5 cm depth in the preceding two days (figures 4(E), (F)). Peak N_2O concentrations in soil air during this period were highest in red clover plots.

Onset of snow cover resulted in an even stronger trend of increasing N_2O and CO_2 concentrations and decreasing O_2 concentrations in the soil air (figures 4(A)–(C)). Cold weather in early January lowered the soil temperature at 5 cm to below −1 °C and frost tube measurements on January 4 and January 16 indicated that frost depth had increased from approximately 2–6 cm to 9–15 cm (figure S.2). Heavy snowfall from 10 to 16 January (figure 3(B)) insulated the soil from further thermal fluctuations. Under snow cover, soil temperature at 5 cm remained at around 0 °C for the remainder of winter, only decreasing briefly around February 8 after partial snowmelt and cold ambient air temperatures.

Starting with the N_2O peak in soil air on December 7 and continuing under deep snow, R-con frequently had higher CO_2 and lower O_2 concentrations in the soil air relative to R-dol (figures 4(B), (C)). This likely reflects the decreased solubility of CO_2 in the more acidic soil of R-con and its impact on the partial pressure of bulk gases such as O_2 (the same trend was seen with N2; not shown).

Most plots reached a maximum in soil air N_2O concentration in January or early February (figure 4(A)). Thereafter subnivean N_2O concentration decreased in all grass-only plots and most red clover plots, while the thick ice layer at the base of the snowpack was still present, likely restricting release of stored N_2O. In most

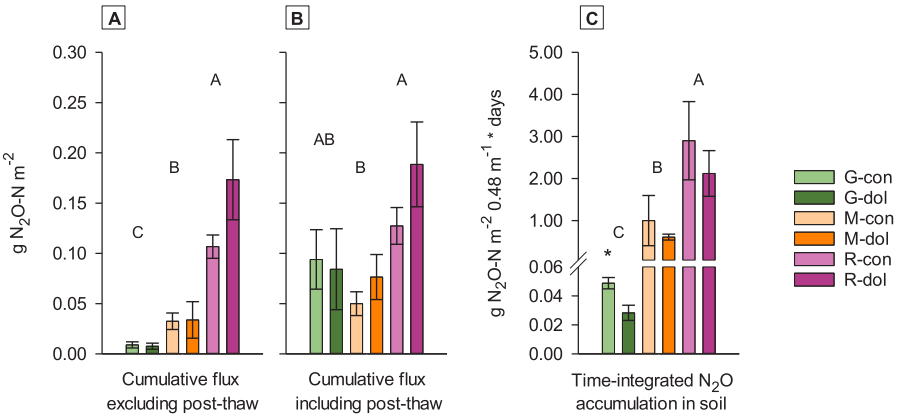


Figure 5. Cumulative N_2O emissions in g N m^{-2} for each treatment throughout the whole study, excluding (A) and including (B) post spring thaw fluxes. Mean (±SE) of 4 replicates. Note that the cumulative fluxes do not include the period of deep snow cover and do not coincide exactly with dates of soil air measurements. (C) Time-integrated N_2O accumulation throughout the whole study in 0–48 cm soil depth in g N m^{-2} * 0.48 m^{-1} * days. Sum of soil air and dissolved fractions. Mean (±SE) of 3 replicates. For each graph, letters indicate Tukey groupings ($p < 0.05$) of species effect on the natural log of the measurement. (*) indicates pH effect on the natural log of the integrals of soil N_2O accumulation (one-sided t test for pure grass, $p < 0.05$).

mixture plots, N_2O concentrations remained stable throughout winter. One R-con and one M-con plot, both in the shaded area of the field, continued increasing subnivean N_2O until peaking just before spring thaw (figure S.4, plots 505 and 521).

During spring thaw, soil air O_2 approached ambient atmospheric concentrations, though soil CO_2 was still elevated in late April (figures 4(B), (C)). On April 4, just before soil air began to re-equilibrate with the atmosphere, average CO_2 concentrations ranged from 49,000 to 75,000 ppm. Concentrations of O_2 ranged from 2 to 5 vol. % on April 4 and tended to be lowest in grass-only plots. From the beginning of spring thaw until later measurements on April 23–28, N_2O concentrations in grass-only plots rose from low, near-atmospheric concentrations to 2–3 ppm, while N_2O in the red clover and mixture plots decreased from their higher winter concentrations to between 2–6 ppm on average—still elevated relative to atmospheric levels (figure 4(A)).

Soil CH_4 concentrations decreased below ambient during periods of reduced soil-atmosphere exchange (figure 4(D)), but peaked above ambient levels in two R-dol plots on April 9–10, just before the final ice melt in spring (figure S.4, plots 211 and 323), indicating net CH_4 production.

By April 23 to 28, after the ground was free of ice and soils dried up, the average N_2O concentrations in grass-only plots increased to the same levels as observed in January under snowpack (figure 4), but were still lower than in red clover plots. Most of the increase occurred at 24 cm below the plough layer (figure S.4, e.g. plot 112). At the same time, the average N_2O concentrations in pure red clover and mixture plots were stable or still decreasing from their higher winter values.

Summed over the top 48 cm of the soil, the maximum observed amount of N_2O-N was much larger in red clover than mixture or grass-only plots. 90 mg N_2O-N m^{-2} 0.48 m^{-1} was recorded in one R-con plot on February 5, which was much more than the peaks for M-con (28 mg N_2O-N m^{-2} 0.48 m^{-1}) or G-con (1 mg N_2O-N m^{-2} 0.48 m^{-1}) plots (data not shown). Limed plots accumulated less N_2O than their respective unlimed control plots: a maximum of 47 mg N_2O-N m^{-2} 0.48 m^{-1} was found in R-dol, 9 mg N_2O-N m^{-2} 0.48 m^{-1} in M-dol, and 0.7 mg N_2O-N m^{-2} 0.48 m^{-1} in G-dol (data not shown).

As mentioned, soil N_2O concentrations varied throughout the period of dense snowpack. Therefore, to compare accumulation of soil N_2O across treatments, we interpolated linearly between sampling days and calculated a plot-wise time integral as g N_2O-N m^{-2} 0.48 m^{-1} days. This metric reflects both magnitude and duration of N_2O accumulation. On average over the whole experiment, this integral was largest in red clover plots, which held about three times the N_2O in the soil as mixture plots, and 65 times that of grass-only plots (figure 5(C)). In the deep snow period, there was a consistent tendency in all sward types that limed plots accumulated less N_2O than control plots integrated over time, although this was statistically significant only for the grass-only plots (figure 6(B)).

Time-integrated N_2O accumulation during the freeze-thaw period in mixture plots was more similar to grass than red clover plots, but under deep snow cover and during spring thaw was more similar to red clover than grass plots (figure 6(B)). During the freeze-thaw period, time-integrated N_2O accumulation in soil of the

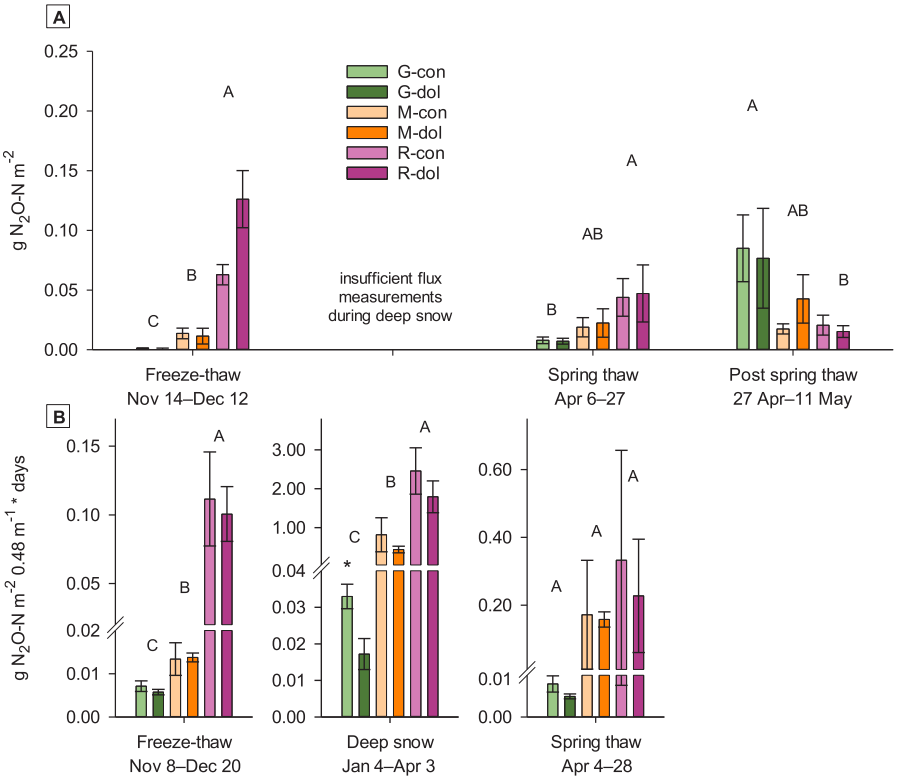


Figure 6. For each seasonal division (not equal in length by days), (A) cumulative N_2O emissions in g N m^{-2}, mean (±SE) of 4 replicates; and (B) Time-integrated N_2O accumulation in 0–48 cm soil depth in g N m^{-2} * 0.48 m^{-1} * days, mean (±SE) of 3 replicates. Letters indicate Tukey groupings ($p < 0.05$) of species effect on the natural log of the measurement. (*) indicates pH effect on the natural log of the integrals of soil N_2O accumulation (one-sided t test for pure grass, $p = 0.05$).

mixture was about one eighth of that in pure red clover, and about twice that of grass-only. During spring thaw, however, mixture plots accumulated almost as much N_2O in the soil as pure red clover, although still significantly less than red clover during the deep snow period (figure 4(A)). Soil N_2O in both clover and mixture plots varied highly during spring thaw. Throughout the deep snow and spring thaw periods, the time-integrated N_2O accumulation in grass-only plots was one order of magnitude less N_2O in the soil than the other treatments.

3.4. Surface N_2O fluxes

Positive N_2O fluxes to the atmosphere prevailed (figure 7(A)). The majority of recorded flux rates were under 50 μg N_2O-N m^{-2} h^{-1}. Peak fluxes occurred on December 7 after a major freeze-thaw near the soil surface (figures 7(B), (C)) and on several dates during spring thaw. Red clover plots reached maximum fluxes between 300 and 1600 μg N_2O-N m^{-2} h^{-1}, and one M-dol plot reached a maximum flux of 785 μg N_2O-N m^{-2} h^{-1}. Grass-only plots had mostly small fluxes, often not significantly different from zero, with occasional larger fluxes not exceeding 111 μg N_2O-N m^{-2} h^{-1}. However, in the post spring thaw period after fertilization, a few grass-only plots and one mixture plot, but no pure red clover plots, showed emissions above 500 μg N_2O-N m^{-2} h^{-1}. One G-dol plot emitted over 1900 μg N_2O-N m^{-2} h^{-1} on May 2, which was the largest flux observed during the experiment. Flux measurements are lacking for the period of deep snow cover.

Fluctuations in flux rates in autumn corresponded roughly with freeze-thaw cycles. From November 10 to 20, diurnal freeze-thaw cycles occurred, whereas from November 20 to 22 the air temperature remained above zero (figure 3(A)). The TDR-thermistor probe placed just below the soil surface indicated soil temperatures around or below 0 °C except for November 23 and December 7 (figure 7(B)), when ambient air temperatures increased. The highest autumn fluxes were observed on December 7, corresponding with a thaw event measured in the afternoon around 2–4 PM. Fluxes were also slightly elevated during thawing on November 23, particularly in grass-only plots; these were measured in the morning around 7–9 AM. Fluxes measured on the days leading

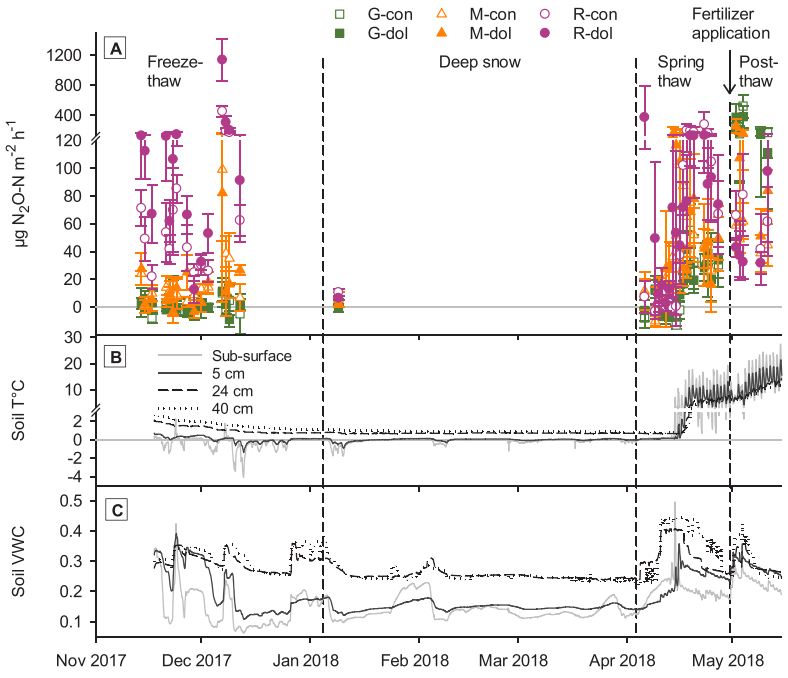


Figure 7. (A) Surface flux of N_2O in $\mu g\ N\ m^{-2}\ h^{-1}$ for each treatment. Daily means (±SE) of 4 replicates. (B) Soil temperature °C and (C) soil VWC at each depth (mean of 4 logging stations). Vertical dashed lines indicate seasonal divisions, and the arrow indicates fertilizer application on April 30 (120 kg N ha^{-1} on grass-only plots, half on mixtures, and none on pure red clover).

up to these two thaw events, while soil was likely frozen, tended to be somewhat smaller (See figure S.5(A) for individual flux measurements in this period). While we did not observe any significant effect of liming on N_2O fluxes during the period of diurnal freeze-thaw in grass or mixture plots, R-dol plots emitted double the cumulative N_2O of R-con plots throughout this period (figure 6(A)), and emitted more than double the N_2O of R-con plots on December 7 (figure 7(A)).

When snow depth was above 10 cm, the autonomous field flux robot vehicle could not drive along its course without removing snow, which would have disrupted the snowpack. The only available flux measurements during the deep snow period, taken on January 9 with light snow cover and frozen soil, showed low flux activity (figures 3(B); 7). Average measured emission rates were 2 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$ in grass-only plots and 8.5 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$ in red clover plots, with mixtures in between (differences between species not significant, figure 7(A)). The pH effect on January 9 was consistent in all species: limed plots emitted ~2 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$ less than control plots on average, though this was not significant ($p=0.12$).

Rapid snowmelt began April 4, but temperatures just below the soil surface did not exceed 1°C before April 14, nor at 5 cm depth before April 15 (figure 7(B)), or April 16 in the shaded area (figure S.3(C)). The soil surface became visible from April 11 onwards and all snow was melted by April 16. Between April 14 and 15, TDR probes showed a sudden increase in soil VWC below the surface and at 5 cm depth (figure 7(C)). Elevated N_2O fluxes were recorded in red clover and clover-grass mixture plots between April 14–17, with emission rates above 200 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$, while grass-only plots remained below 50 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$. However, towards the end of spring thaw on April 19–27, grass-only plots emitted between 50 and 110 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$, more than observed at any other time in grass-only plots.

Post spring thaw, from May 1 to 5, the average soil temperature at 5 cm depth increased by about 6° C (figure 7(B)) and 22 mm precipitation increased soil VWC (figure 7(C)). Fertilizer was applied on April 30 (120 kg N ha^{-1} in grass, half dose N in mixtures, and none in pure red clover). From May 2 to 11, N_2O emissions increased in grasses and decreased in pure clover (figure 7(A)). N_2O fluxes were highest in grass-only plots, with 10 measurements above 500 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$, and maximum fluxes up to 1900 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$. Pure red clover plots emitted at most 250 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$. Mixture plots were in between, with a few measurements between 500 and 900 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$.

In both fall and spring, there were instances of near-zero or even negative N_2O fluxes (average −30, at most −62 μg N_2O-N m^{-2} h^{-1}). We found no correlation between ambient air temperature and negative N_2O fluxes, which occurred from +5 to +22°C. While we observed negative fluxes in grass and mixture plots during all time periods, we only observed negative fluxes in pure red clover plots in spring (figure 7(A)). In most cases the springtime negative fluxes, regardless of treatment, corresponded to ambient or below-ambient N_2O concentrations in the soil air (figure S.4), indicating that there was net N_2O uptake from the atmosphere. We did not observe below-ambient soil air N_2O in the autumn.

We interpolated between individual measurements to estimate cumulative flux (figures 5(A); 6(A)); this did not include dates between December 12 and April 6. Over the whole experiment, pure red clover plots had the highest cumulative N_2O emissions. From freeze-thaw to spring thaw, but excluding 'post spring thaw' measurements in May, red clover plots emitted on average 0.14 g N m^{-2}, about four times the emission in mixture plots and nearly seventeen times the emission in grass-only plots (figure 5(A)). The stimulatory effect of red clover on N_2O emissions was strongest during the freeze-thaw period in autumn and early winter, with red clover emitting more than seven times more N_2O than mixture plots and 120 times more than grass-only plots (figure 6(A)). We observed the same pattern during spring thaw, but the differences between species were smaller and emissions in mixture plots were not significantly different from pure red clover or grass-only plots (figure 6(A)). Post spring thaw, red clover plotsemitted less than one quarter of the cumulative N_2O emitted in the fertilized grass-only plots (figure 6(A)). This late burst of N_2O emissions in grass-only plots meant that over the whole experiment from fall to post spring thaw, grass-only plots emitted slightly more N_2O than mixture plots (not significant, figure 5(B)).

We did not find any statistically significant pH effect on N_2O fluxes when cumulated over the entire experimental period, irrespective of sward type. However, in the period of freeze-thaw cycles in autumn, limed red clover plots had almost twice the cumulative emissions as control red clover plots and this effect was significant (figure 6(A)). Furthermore, the ANOVA model for the freeze-thaw period indicated significant interaction between pH and red clover on fluxes ($p<0.05$), i.e. there was a uniquely different pH effect in red clover plots than in grass-only or mixture plots.

3.5. Effect of mixtures on N_2O fluxes

In the grass-clover mixture, the magnitudes of cumulative N_2O emissions were consistently in between those of grass-only and pure clover. However, this relationship was not proportional to the amount of clovers present in the mixture, suggesting some diversity effect on off-season N_2O production. M-con plots contained 49% red clover, and M-dol 38% by DM weight harvested in September 2017 (table 1(A)). We calculated the 'expected N_2O emissions' in the mixtures assigning average N_2O emission per DM measured in pure grass and clover stands to the proportions of grass in clover in the mixtures separately for each liming treatment (equation (3); table S.4). Due to large variation, differences between treatments were not statistically significant, but showed a trend that mixtures emitted less N_2O than expected. Cumulative emissions in M-con were on average 30% of those expected during the freeze-thaw period, and 50% of those expected during spring thaw. Likewise, M-dol emitted 19% of the N_2O emission expected during freeze-thaw, but during spring thaw, nearly as much (79%) as expected. Post spring thaw and after spring fertilization (120 kg N ha^{-1} in grass, half dose N in mixtures, and none in pure red clover), when grass plots dominated N_2O emissions, M-con plots again emitted 30% of the N_2O that would be expected from the share of clover, while M-dol emitted 86% of expected. This trend remained unchanged post spring thaw when removing two outliers with high Min-N and high post spring thaw fluxes (plots 628 and 527, table S.4).

4. Discussion

4.1. Soil air concentrations and emissions of N_2O

It is well known that over-winter N_2O emissions in temperate and boreal soils can make up a large part of the annual greenhouse gas budget of crop production (Christensen and Tiedje 1990, Flessa *et al* 1995) including that of perennial forage crops (Kaiser *et al* 1998). In Norway, 65% of all cropland is managed grassland, often heavily fertilized 2–3 times per year for forage production (Hansen *et al* 2014). Compared to annual croplands, grasslands across Europe (at sites further south than our study) were found to have higher variability of N_2O emissions, especially when fertilized intensively (Rees *et al* 2013). Peak N_2O fluxes measured in our grass-clover mixture were similar to those measured by Hansen *et al* (2014) in a similar mixture during the growing season in western Norway, around 100 μg N_2O-N m^{-2} h^{-1}. A few fluxes we measured from mixture and pure clover plots exceeded this by an order of magnitude. Some of our grass-only plots also reached fluxes of over 100 μg N_2O-N m^{-2} h^{-1} immediately after spring fertilization. Results from Hansen *et al* (2014) also indicated that growing-season N_2O emissions associated with pronounced drying-rewetting during a year undergoing drought, were

positively correlated with the fraction of clover in the ley, whereas there was no relationship with clover in a non-drought year. While we are not aware of any whole-year N_2O emissions studies in Norwegian grasslands which quantify winter N_2O emissions in the context of an annual budget, our study demonstrates that N_2O is produced in soil both during diurnal soil freezing-thawing, and under prolonged snow cover at soil temperatures near the freezing point, and that this effect is pronounced in clover-containing swards.

N_2O produced over-winter is either released instantly as was the case during periods of early winter freeze-thaw and spring thaw, or is trapped under frozen soil and/or surface ice and snowpack. The resulting soil-atmosphere flux dynamics are difficult to interpret and highly dependent on diffusion conditions, shifting diurnally and seasonally. Still, in periods where we measured both soil air and surface flux, the relationship was consistent within each sward type, i.e. treatments with large N_2O concentrations in soil air also had large fluxes. In some instances, the relationship between soil air and fluxes was decoupled, for example during freeze-thaw when R-dol emitted more N_2O than R-con despite equal accumulation of N_2O in the soil (figures 7(A); 4(A)). The latter indicates that N_2O was produced in the uppermost centimeters of the soil and diffused immediately to the atmosphere.

Similar to findings of two long-term measurement campaigns by Wagner-Riddle *et al* (2017) on cropland in Canada, N_2O emissions were low during the frozen soil phase of freeze-thaw cycles, while peak emissions occurred during thaw events. Relating spring thaw N_2O emissions to release of N_2O previously accumulated under snow or ice cover is not straightforward. Our soil air observations showed that many plots reached peak accumulation of N_2O long before the onset of spring thaw, suggesting 'leakage' of accumulated N_2O through the snowpack or reuptake and reduction to N_2 by denitrification. The latter process is plausible under prolonged periods of anoxia when N_2O becomes the only available N oxyanion for denitrification. Similar to our results, Wagner-Riddle *et al* (2017) reported low N_2O emissions during periods of prolonged snowpack. It was impossible to retrieve soil samples from under the ice layer during the period of snow cover, but it is reasonable to assume that NO_3^- was depleted and therefore N_2O the only electron acceptor for denitrification. The two R-dol plots which accumulated above-ambient CH_4 concentrations in soil air just before spring thaw, indicating a very reductive soil environment supportive of methanogenesis, indeed appear to have consumed N_2O by early February (figure S.4, plots 211 and 323). N_2O loss by downward gaseous diffusion in the soil profile was likely restricted by high BD, leaving an effective porosity of only around 20 vol. %.

Irrespective of the subnivean N_2O dynamics, accumulated N_2O is unlikely to entirely account for spring thaw emissions. For example, M-dol plot 527 accumulated a maximum of 7.4 mg N_2O-N m^{-2} 0.48 m^{-1} in soil air on April 4, but had a cumulative spring thaw flux of 12.6 mg N_2O-N m^{-2}, suggesting that at least 40% of the emitted N_2O was created *de novo*. Thus, both release from winter accumulation and new production seem to be important for spring thaw emissions.

4.2. Effect of clover on off-season N_2O

The results support our hypothesis (1) that red clover significantly stimulates off-season N_2O production, during freeze-thaw cycles in uncovered soil in late fall, throughout winter when soil is covered with snow and ice, and during spring thaw. This stimulation of off-season N_2O production reflects the addition of N-rich litter from frost vulnerable clover tissues (Sturite *et al* 2007a, 2007b), with no actively growing plants competing for the N_{min} released by mineralization and nitrification of the labile organic N. This was also seen in the N_{min} values on December 8 after diurnal freeze-thaws; N_{min} values were low for all treatments (table 1(C)), but slightly elevated in red clover plots (significant for R-dol) which went along with highest N_2O emission in clovers recorded during this period (figure 6(A)). Also, during freeze-thaw and especially under snow cover, pure red clover plots had significantly more time-integrated N_2O accumulation than other sward types (figure 6(B)). The transition to warmer temperatures towards the end of April, after the disappearance of ice but before spring fertilization, brought decreasing N_2O fluxes in clovers (figure 7(A)). This could mean that clover biomass available for decomposition had become depleted over winter or that greening clover competed well for soil mineral N.

In grass plots, N_2O fluxes and accumulation of N_2O in soil (figures 7(A); 4(A)) increased after the disappearance of ice and before spring fertilization, yet remained lower than in clovers. It is possible that grass residues from the previous season continued decomposing into the spring whereas clover residues were already depleted. We also noted that spring regrowth in grass-only and mixture plots commenced earlier than in pure red clover. It is therefore possible that input of root exudates from actively-growing grasses triggered larger emissions in grass plots through directly providing labile N and C to nitrification and denitrification or indirectly through priming SOM decomposition, while depleting soil O_2. After spring N fertilization, grasses became a larger source of N_2O than clovers, which had not received any extraneous N (figures 6(A); 5(B)), and also than the mixture, which had received half the N dose.

Using a simple calculation, we estimated that the N_2O saved in reducing mineral fertilizer by including clovers may be at least partially offset by off-season N_2O emission in clovers. We converted observed cumulative off-season N_2O emissions to their fertilizer equivalent, assuming an N_2O emission factor of 1.6% for mineral fertilizer in wet climates from IPCC (2019). Fluxes during our freeze-thaw and spring thaw periods, i.e. before spring fertilization, contributed additional N_2O equivalent to 61 kg N ha^{-1} yr^{-1} fertilizer addition in R-con plots and 104 kg N ha^{-1} yr^{-1} fertilizer addition in R-dol plots. Thus although pure red clover plots were not fertilized, R-dol emitted off-season nearly 40% of the annual N_2O expected from an application of 270 kg N ha^{-1} yr^{-1}, the level at which grass-only plots were fertilized. Off-season N_2O emissions from grass-clover mixture plots, amounted to a fertilizer equivalent of 15–16 kg N ha^{-1} yr^{-1}, or about 11% more than the 140 kg N ha^{-1} yr^{-1} applied to these plots.

4.3. Mitigation effect by mixtures?

Although our mixtures produced more off-season N_2O than grass-only swards, they theoretically still had a lower annual N_2O footprint and supposedly less winter NO_3^- leaching (Elgersma *et al* 1998) owing to half-dose N fertilization. Further, the nutritional forage quality of the mixture measured by N yield m^{-2} may have been higher than in pure grass. Although we did not measure N content in biomass from this field, it is known that grass-clover mixtures can overyield N relative to their proportion of clovers (Nyfeler *et al* 2011).

Beyond these annual diversity effects, which may justify slightly increased off-season N_2O, our data indicated an interesting diversity effect on off-season N_2O emissions. During the freeze-thaw period, mixtures emitted less N_2O than would be expected from their DM proportion of clover to grass (table S.4). This trend was weaker but still present during spring thaw. This suggests that diversity effects in grass-clover mixtures in principal also affect off-season N_2O emissions. Research on the ideal proportions of grass to clover for N_2O mitigation has so far not shown a clear relationship between proportion and annual N_2O emissions (Fuchs *et al* 2020); note however that most of these experiments were carried out in temperate locations. Varying mineral N application levels also complicate experimental designs and interpretation of these results; still, modeling by Fuchs *et al* (2020) demonstrated that replacing fertilizer with legumes reduced N_2O emissions while maintaining productivity.

Post-spring thaw, after spring fertilization and when grass-only plots emitted much more N_2O than pure red clovers, M-con again emitted less N_2O than would be expected, while M-dol showed little mitigation effect. Noting also that the variation of cumulative post-spring thaw flux in M-con was lower than in M-dol (figure 6(A)), increased N cycling in limed plots after fertilization may have confounded an N_2O mitigating diversity effect in this case.

4.4. Liming effect

The results did not support our hypothesis (2) that liming reduces off-season N_2O accumulation and emission in clovers by favoring more complete denitrification of N_2O to N_2. Much to the contrary, R-dol plots emitted double the cumulative N_2O flux as R-con throughout the freeze-thaw period in fall (figure 6(A)). This might reflect overall larger N turnover in limed than unlimed clover plots after initial frost killing; R-dol had slightly higher yields in September than R-con (tables 1(A), (C); not significant). Nitrification is strongly stimulated by high pH (Parton *et al* 2001) and liming likely supported higher rates of nitrification than in the control soil, implicating nitrification or coupled nitrification-denitrification (Kremen *et al* 2005) as the dominant source of N_2O early in winter. Soil samples taken on December 8, one day after peaking freeze-thaw induced N_2O fluxes, showed that R-dol had significantly more extractable NO_3^- than R-con (table 1(C)). Raising pH may also favor ammonia oxidizing bacteria which produce more N_2O than ammonia oxidizing archaea (Hink *et al* 2018), although this increase in N_2O is theoretically lower in magnitude than the decrease of N_2O produced by denitrification.

If N_2O is produced in the uppermost soil, both nitrification and denitrification can be a source, as long as some local anoxia exists. Given an O_2 concentration in soil air which could still support nitrification throughout the freeze-thaw period (minimum 17% in R-dol and 14% in R-con; figure 4(B)) and low soil VWC in the topsoil (figure 4(F)), coupled nitrification-denitrification may have occurred in medium-sized soil aggregates (Kremen *et al* 2005). Song *et al* (2017) note that while freeze-thaw may inhibit nitrification in laboratory experiments, field experiments have shown freeze-thaw to stimulate nitrification. Although we did not measure mineral NO_2^- in our soil (the samples were sieved and frozen a few hours after collection), transient nitrite accumulation could be another potent inducer of N_2O emissions (Giguere *et al* 2017), potentially also off-season (Venterea *et al* 2020).

During the period of deep snow cover, R-dol plots tended to have less time-integrated N_2O accumulation in soil than R-con plots (not significant, $p=0.41$), indicating that liming might reduce longer-term N_2O accumulation in the soil. Since anoxic conditions under prolonged snowpack do not support nitrification, denitrification was likely prevailing, mediating a pH effect through pH control of N_2O reductase activity.

However, this effect may be modulated by acid-tolerant complete denitrifiers proliferating during extended periods of anoxia, especially when nitrogenous electron acceptors other than N_2O become scarce (Palmer *et al* 2010). R-con showed especially high variability in N_2O concentrations in the soil air during spring thaw, which highlights the potential risk that unlimed clover-containing plots can produce large quantities of N_2O through denitrification in spring, even if such large quantities were not observed consistently.

The clearest evidence for a possible mitigation effect of off-season N_2O by liming was seen in grass-only plots during the deep snow-covered period, in which limed plots had significantly less time-integrated N_2O accumulation in soil air relative to the control (figure 6(B)). The fact that this effect was not seen in clover plots suggests that decomposition of N-rich clover substrates overrides the mitigation effect by liming.

4.5. Conclusions

While many studies on N_2O from agricultural systems focus on the growing season, the body of literature about off-season emissions is increasing. Studies investigating the N_2O effect of including legumes, whether in grassland or cover cropping systems, highlight the importance of accounting for winter in annual N_2O budgets. Our study details dynamics of subnivean N_2O accumulation and flux and compares them among distinct grassland communities throughout variable winter conditions: repeated freeze-thaws of exposed soil, and trapped under frozen soil.

Our data point at a tradeoff between including clover in a grass mixture for saving N fertilizer in the summer and inducing extra N_2O emissions in winter. From the perspective of N use efficiency, if stands of pure clover and grass-clover mixture yield more N in forage than grass-only stands, or if grass-clover mixtures can reduce N leaching, this may partially justify additional N_2O emissions, especially if mineral N additions to clover-containing swards can be reduced.

Liming may enhance winter emissions of N_2O produced *de novo* in topsoil from decaying N-rich substrates, such as clovers. There is some evidence, however, that over long periods of reduced exchange between soil and atmosphere, favoring denitrification but not nitrification, liming may reduce N_2O accumulation, likely by supporting N_2O reductase activity.

Off-season, the N_2O risk posed by pure clover swards seemingly overrides the advantage of liming seen in grass swards. However, there was some evidence that our grass-clover mixtures, containing around 40%–50% clover by DM yield, may emit less N_2O than might be predicted from the component species. Further studies on this potential diversity effect on N_2O mitigation would be beneficial for developing planting guidelines in areas experiencing winter conditions with freeze-thaw. In addition, it may be worthwhile to test the effect of more winter-hardy legume species. Selecting species which grow late into the season and absorb and store excess N_{min} belowground, as in the practice of using 'catch crops,' may help insofar as they can resist decomposition under long and harsh winter conditions.

Acknowledgments

Erin Byers was funded by the Faculty for Environmental Sciences and Natural Resource Management at the Norwegian University of Life Sciences. Marina A. Bleken and Peter Dörsch were funded by the FACCE ERA-GAS projects 'ResidueGas' and 'MAGGE-pH', respectively (both under the ERA-GAS grant agreement ID 696356). We thank Trygve Fredriksen, Øyvind Peder Vartdal, Toril Trædal, and Prashanta Raut (all NMBU) for assistance in the field.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

ORCID iDs

Erin Byers https://orcid.org/0000-0002-6749-7264
Marina A Bleken https://orcid.org/0000-0003-0892-1157
Peter Dörsch https://orcid.org/0000-0002-4916-1839

References

Bakken L R, Bergaust L, Liu B and Frostegård Å 2012 Regulation of denitrification at the cellular level: a clue to the understanding of N_2O emissions from soils *Philos. Trans. R. Soc. B Biol. Sci.* **367** 1226–34

Basche A D, Miguez F E, Kaspar T C and Castellano M J 2014 Do cover crops increase or decrease nitrous oxide emissions? A meta-analysis *J. Soil Water Conserv.* **69** 471–82
Burton D L and Beauchamp E G 1994 Profile nitrous oxide and carbon dioxide concentrations in a soil subject to freezing *Soil Sci. Soc. Am. J.* **58** 115
Carter M S and Ambus P 2006 Biologically fixed N2 as a source for N_2O production in a grass–clover mixture *Measured by 15N2. Nutr. Cycl. Agroecosystems* **74** 13–26
Christensen S and Christensen B T 1991 Organic matter available for denitrification in different soil fractions: effect of freeze/thaw cycles and straw disposal *J. Soil Sci.* **42** 637–47
Christensen S and Tiedje J M 1990 Brief and vigorous N_2O production by soil at spring thaw *J. Soil Sci.* **41** 1–4
Congreves K A, Wagner-Riddle C, Si B C and Clough T J 2018 Nitrous oxide emissions and biogeochemical responses to soil freezing-thawing and drying-wetting *Soil Biol. Biochem.* **117** 5–15
Cowan N J, Famulari D, Levy P E, Anderson M, Bell M J, Rees R M, Reay D S and Skiba U M 2014 An improved method for measuring soil N_2O fluxes using a quantum cascade laser with a dynamic chamber: dynamic chamber method *Eur. J. Soil Sci.* **65** 643–52
Doane T A and Horwáth W R 2003 Spectrophotometric determination of nitrate with a single reagent *Anal. Lett.* **36** 2713–22
Elgersma A, Schlepers H, Stegeman A G and Kool A 1998 Nitrate leaching under grass-clover mixtures *Proc. 16th General Meeting European Grassland Federation: Grassland and Land Use Systems* 1 (*Grassland Science in Europe*) *(Grado, Italy, 1996)* ed G. Parente
Flessa H, Dörsch P and Beese F 1995 Seasonal variation of N_2O and CH4 fluxes in differently managed arable soils in southern Germany *J. Geophys. Res.* **100** 23115
Fowler D *et al* 2013 The global nitrogen cycle in the twenty-first century *Philos. Trans. R. Soc. B Biol. Sci.* **368** 20130164
Freibauer A and Kaltschmitt M 2003 Controls and models for estimating direct nitrous oxide emissions from temperate and sub-boreal agricultural mineral soils in Europe *Biogeochemistry* **63** 93–115
Fuchs K *et al* 2020 Evaluating the potential of legumes to mitigate N_2O emissions from permanent grassland using process-based models *Glob. Biogeochem. Cycles* **34** 1–36
Gabriel J L, Alonso-Ayuso M, García-González I, Hontoria C and Quemada M 2016 Nitrogen use efficiency and fertiliser fate in a long-term experiment with winter cover crops *Eur. J. Agron.* **79** 14–22
Garten C T Jr, Smith J L, Tyler D D, Amonette J E, Bailey V L, Brice D J, Castro H F, Graham R L, Gunderson C A and Izaurralde R C 2010 Intra-annual changes in biomass, carbon, and nitrogen dynamics at 4-year old switchgrass field trials in west Tennessee, USA *Agric. Ecosyst. Environ.* **136** 177–84
Giguere A T, Taylor A E, Suwa Y, Myrold D D and Bottomley P J 2017 Uncoupling of ammonia oxidation from nitrite oxidation: impact upon nitrous oxide production in non-cropped Oregon soils *Soil Biol. Biochem.* **104** 30–8
Graham L and Risk D 2018 Explaining CO_2 fluctuations observed in snowpacks *Biogeosciences* **15** 847–59
Griffis T J, Chen Z, Baker J M, Wood J D, Millet D B, Lee X, Venterea R T and Turner P A 2017 Nitrous oxide emissions are enhanced in a warmer and wetter world *Proc. Natl Acad. Sci.* **114** 12081–5
Groffman P M, Driscoll C T, Fahey T J, Hardy J P, Fitzhugh R D and Tierney G L 2001 Colder soils in a warmer world: a snow manipulation study in a northern hardwood forest ecosystem *Biogeochemistry* **56** 135–50
Hansen S, Bernard M-E, Rochette P, Whalen J K and Dörsch P 2014 Nitrous oxide emissions from a fertile grassland in Western Norway following the application of inorganic and organic fertilizers *Nutr. Cycl. Agroecosystems* **98** 71–85
Hensen A, Groot T T, van den Bulk W C M, Vermeulen A T, Olesen J E and Schelde K 2006 Dairy farm CH4 and N_2O emissions, from one square metre to the full farm scale *Agric. Ecosyst. Environ.* **112** 146–52
Hink L, Gubry-Rangin C, Nicol G W and Prosser J I 2018 The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions *ISME J* **12** 1084–93
IPCC 2019 Chapter 11: N_2O emissions from managed soils, and CO2 emissions from lime and urea application *2019 Refinement to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories* ed E Calvo Buendia *et al* (IPCC) *(Switzerland)*
IPCC 2014 *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* ed R K Pachauri and L A Meyer (IPCC) *(Geneva, Switzerland)*
IUSS Working Group WRB 2015 World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps *World Soil Resources Reports No. 106* (Rome: FAO)
Kaiser E-A, Kohrs K, Kücke M, Schnug E, Munch J C and Heinemeyer O 1998 Nitrous oxide release from arable soil: importance of perennial forage crops *Biol. Fertil. Soils* **28** 36–43
Kim D-G, Vargas R, Bond-Lamberty B and Turetsky M R 2012 Effects of soil rewetting and thawing on soil gas fluxes: a review of current literature and suggestions for future research *Biogeosciences* **9** 2459–83
Koponen H T and Martikainen P J 2004 Soil water content and freezing temperature affect freeze–thaw related N_2O production in organic soil *Nutr. Cycl. Agroecosystems* **69** 213–9
Kremen A, Bear J, Shavit U and Shaviv A 2005 Model demonstrating the potential for coupled nitrification denitrification in soil aggregates *Environ. Sci. Technol.* **39** 4180–8
Krom M D 1980 Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate *Analyst* **105** 305
Leimer S, Oelmann Y, Wirth C and Wilcke W 2015 Time matters for plant diversity effects on nitrate leaching from temperate grassland *Agric. Ecosyst. Environ.* **211** 155–63
Liu B, Frostegård Å and Bakken L R 2014 Impaired reduction of N_2O to N_2 in acid soils is due to a posttranscriptional interference with the expression of nosZ *mBio* **5** e01383-14-e01383-14
Maljanen M, Kohonen A-R, Virkajärvi P and Martikainen P J 2007 Fluxes and production of N_2O, CO_2 and CH_4 in boreal agricultural soil during winter as affected by snow cover *Tellus B Chem. Phys. Meteorol.* **59** 853–9
McCool D K and Molnau M 1984 Measurement of frost depth *Proc. Western Snow Conf.* 52, pp 33–42
Monson R K, Burns S P, Williams M W, Delany A C, Weintraub M and Lipson D A 2006 The contribution of beneath-snow soil respiration to total ecosystem respiration in a high-elevation, subalpine forest: BENEATH-SNOW SOIL RESPIRATION *Glob. Biogeochem. Cycles* **20** GB3030
Mørkved P T, Dörsch P and Bakken L R 2007 The N_2O product ratio of nitrification and its dependence on long-term changes in soil pH *Soil Biol. Biochem.* **39** 2048–57
Mørkved P T, Dörsch P, Henriksen T M and Bakken L R 2006 N_2O emissions and product ratios of nitrification and denitrification as affected by freezing and thawing *Soil Biol. Biochem.* **38** 3411–20
Müller C, Martin M, Stevens R, Laughlin R, Kammann C, Ottow J C and Jäger H-J 2002 Processes leading to N_2O emissions in grassland soil during freezing and thawing *Soil Biol. Biochem.* **34** 1325–31

Nadeem S, Børresen T and Dörsch P 2015 Effect of fertilization rate and ploughing time on nitrous oxide emissions in a long-term cereal trail in south east Norway *Biol. Fertil. Soils* **51** 353–65
Nadeem S, Hansen S, Azzaroli Bleken M and Dörsch P 2012 N_2O emission from organic barley cultivation as affected by green manure management *Biogeosciences* **9** 2747–59
Nyfeler D, Huguenin-Elie O, Suter M, Frossard E and Lüscher A 2011 Grass–legume mixtures can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic sources *Agric. Ecosyst. Environ.* **140** 155–63
Öquist M G, Nilsson M, Sörensson F, Kasimir-Klemedtsson Å, Persson T, Weslien P and Klemedtsson L 2004 Nitrous oxide production in a forest soil at low temperatures—processes and environmental controls *FEMS Microbiol. Ecol.* **49** 371–8
Østrem L, Rapacz M, Larsen A, Dalmannsdottir S and Jørgensen M 2015 Influences of growth cessation and photoacclimation on winter survival of non-native Lolium–Festuca grasses in high-latitude regions *Environ. Exp. Bot.* **111** 21–31
Palmer K, Drake H L and Horn M A 2010 Association of novel and highly diverse acid-tolerant denitrifiers with N_2O fluxes of an acidic fen *Appl. Environ. Microbiol.* **76** 1125–34
Parton W J, Holland E A, Del Grosso S J, Hartman M D, Martin R E, Mosier A R, Ojima D S and Schimel D S 2001 Generalized model for NOx and N_2O emissions from soils *J. Geophys. Res. Atmospheres* **106** 17403–19
Priemé A and Christensen S 2001 Natural perturbations, drying–wetting and freezing–thawing cycles, and the emission of nitrous oxide, carbon dioxide and methane from farmed organic soils *Soil Biol. Biochem.* **33** 2083–91
Ravishankara A R, Daniel J S and Portmann R W 2009 Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century *Science* **326** 123–5
Rees R M *et al* 2013 Nitrous oxide emissions from European agriculture—an analysis of variability and drivers of emissions from field experiments *Biogeosciences* **10** 2671–82
Risk N, Snider D and Wagner-Riddle C 2013 Mechanisms leading to enhanced soil nitrous oxide fluxes induced by freeze–thaw cycles *Can. J. Soil Sci.* **93** 401–14
Risk N, Wagner-Riddle C, Furon A, Warland J and Blodau C 2014 Comparison of simultaneous soil profile N_2O concentration and surface N_2O flux measurements overwinter and at spring thaw in an agricultural soil *Soil Sci. Soc. Am. J.* **78** 180–93
Rochette P and Janzen H H 2005 Towards a revised coefficient for estimating N_2O emissions from legumes *Nutr. Cycl. Agroecosystems* **73** 171–9
Ruan L and Robertson G P 2017 Reduced snow cover increases wintertime nitrous oxide (N_2O) emissions from an agricultural soil in the upper US Midwest *Ecosystems* **20** 917–27
Russenes A L, Korsaeth A, Bakken L R and Dörsch P 2016 Spatial variation in soil pH controls off-season N_2O emission in an agricultural soil *Soil Biol. Biochem.* **99** 36–46
Russenes A L, Korsaeth A, Bakken L R and Dörsch P 2019 Effects of nitrogen split application on seasonal N_2O emissions in southeast Norway *Nutr. Cycl. Agroecosystems* **115** 41–56
Röver M, Heinemeyer O and Kaiser E-A 1998 Microbial induced nitrous oxide emissions from an arable soil during winter *Soil Biol. Biochem.* **30** 1859–65
Sander R 2015 Compilation of Henry's law constants (version 4.0) for water as solvent *Atmospheric Chem. Phys.* **15** 4399–981
Sehy U, Dyckmans J, Ruser R and Munch J C 2004 Adding dissolved organic carbon to simulate freeze-thaw related N_2O emissions from soil *J. Plant Nutr. Soil Sci.* **167** 471–8
Sommerfeld R A, Mosier A R and Musselman R C 1993 CO_2, CH_4 and N_2O flux through a Wyoming snowpack and implications for global budgets *Nature* **361** 140–2
Song Y, Zou Y, Wang G and Yu X 2017 Altered soil carbon and nitrogen cycles due to the freeze-thaw effect: a meta-analysis *Soil Biol. Biochem.* **109** 35–49
Sturite I, Henriksen T M and Breland T A 2007a Longevity of white clover (Trifolium repens) leaves, stolons and roots, and consequences for nitrogen dynamics under northern temperate climatic conditions *Ann. Bot.* **100** 33–40
Sturite I, Henriksen T M and Breland T A 2007b Winter losses of nitrogen and phosphorus from Italian ryegrass, meadow fescue and white clover in a northern temperate climate *Agric. Ecosyst. Environ.* **120** 280–90
Teepe R, Brumme R and Beese F 2001 Nitrous oxide emissions from soil during freezing and thawing periods *Soil Biol. Biochem.* **33** 1269–75
Tian H *et al* 2019 Global soil nitrous oxide emissions since the preindustrial era estimated by an ensemble of terrestrial biosphere models: Magnitude, attribution, and uncertainty *Glob. Change Biol.* **25** 640–59
Tzanakakis V, Taylor A E and Bottomley P J 2020 Impact of freeze-thaw on the contributions of AOA and AOB to N-flush induced nitrification in meadow soils *Soil Biol. Biochem.* 108015
Ussiri D and Lal R 2013 Global sources of nitrous oxide *Soil Emission of Nitrous Oxide and Its Mitigation* ed D Ussiri and R Lal (Netherlands, Dordrecht: Springer) pp 131–75
Venterea R T, Coulter J A and Clough T J 2020 Nitrite accumulation and nitrogen gas production increase with decreasing temperature in urea-amended soils: experiments and modeling *Soil Biol. Biochem.* **142** 107727
Virkajärvi P, Maljanen M, Saarijärvi K, Haapala J and Martikainen P J 2010 N_2O emissions from boreal grass and grass - clover pasture soils *Agric. Ecosyst. Environ.* **137** 59–67
Vitousek P M, Aber J D, Howarth R W, Likens G E, Matson P A, Schindler D W, Schlesinger W H and Tilman D G 1997 Technical report: human alteration of the global nitrogen cycle: sources and consequences *Ecol. Appl.* **7** 737
Wagner-Riddle C, Congreves K A, Abalos D, Berg A A, Brown S E, Ambadan J T, Gao X and Tenuta M 2017 Globally important nitrous oxide emissions from croplands induced by freeze–thaw cycles *Nat. Geosci.* **10** 279–83
Woledge J, Tewson V and Davidson I A 1990 Growth of grass/clover mixtures during winter *Grass Forage Sci.* **45** 191–202
Wolff M, Thue-Hansen V and Grimenes A A 2018 *Meteorologiske data for Ås 2017* Feltstasjon for bioklimatiske studier, Soås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet (https://doi.org/ISBN 978-82-7636-031-8)
Wolff M, Thue-Hansen V and Grimenes A A 2019 *Meteorologiske data for Ås 2018* Feltstasjon for bioklimatiske studier, Soås, Fakultet for realfag og teknologi, Norges Miljø- og Biovitenskapelige Universitet (https://doi.org/ISBN 978-82-7636-032-5)

Winter N_2O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH

Erin Byers, Peter Dörsch, Susanne Eich-Greatorex, Marina A. Bleken

Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Supplementary materials

Table S.1: Average soil properties in the experimental field (means ±SE). Soil samples for chemical and texture analyses were taken in 2012 from 0-20 cm depth, and in 2014 from 20-35 and 35-50 cm depths. Soil samples for physical analyses were taken in 2014 from 10-15, 25-30, and 40-45 cm depths.

Depth	% Total C	% Total N	% Clay	% Silt	% Sand
0-20 cm	2.88 ±0.04	0.26 ±0.004	27.2 ±0.4	47.9 ±0.7	24.8 ±1.0
20-35 cm	0.99 ±0.08	0.10 ±0.01	25.9 ±0.6	42.3 ±0.9	31.7 ±1.5
35-50 cm	0.48 ±0.02	0.07 ±0.00	28.4 ±0.4	53.5 ±0.3	18.2 ±0.4

	Bulk density g cm^{-3}	Porosity vol %	Particle density g cm^{-3}	Water vol % at 100 kPa	Water vol % at 1500 kPa (permanent wilting point)
10-15 cm	1.18 ±0.02	53.8 ±0.7	2.55 ±0.02	30.7 ±0.5	13.0 ±0.3
25-30 cm	1.53 ±0.02	42.9 ±0.7	2.68 ±0.00	31.9 ±0.6	17.8 ±0.5
40-45 cm	1.66 ±0.03	38.5 ±1.1	2.69 ±0.01	28.5 ±1.3	16.9 ±0.9

Table S.2: Species and cultivars and seeding percentages by weight in grass-only (G), grass-red clover mixture (M), and pure red clover (R) swards.

Sward type	G	M	R
Timothy: *Phleum pratense* L. cv. Grindstad	20	17	
Perennial ryegrass: *Lolium perenne* L. cv. Figgjo	25	21	
Meadow fescue: *Schedonorus pratensis* (Huds.) P.Beauv. cv. Fure	25	21	
Tall fescue: *Schedonorus arundinaceus* (Schreb.) Dumort. cv. Swaj	20	17	
Common meadow grass: *Poa pratensis* L. cv. Knut	10	9	
Red clover: *Trifolium Pratense* L. cv. Lea	0	15	100%

Table S.3: Sampling dates of soil air and surface fluxes according to seasonal divisions as determined by weather conditions. Soil air sampling was discontinued after 28 April, but additional surface flux measurements were taken in early May. (*) – Denotes 2 to 3 rounds of flux measurements per day.

Seasonal division	Soil air samples collected	Surface fluxes measured
Freeze-thaw during late fall, early winter	Nov 8, 15, 22, 29 Dec 7, 12, 20	Nov 14, 15, 17, 21, 22, 23, 24, 27, 29 Dec 1, 3, 7, 8, 9, 12
Deep snow cover	Jan 4, 15-16, 25 Feb 5, 13, 21 Mar 6, 13, 21, 26	Jan 9 only
Spring thaw	Apr 4, 9-10, 16, 23, 28	Apr 6, 9, 10, 11, 12, 13*, 14*, 15, 16, 17*, 18, 19, 20, 23, 24, 25, 27
Post spring thaw		May 2*, 3, 4*, 9, 11

Table S.4 (following pages): Detailed data for individual plots. Yields are from the last harvest before the experiment on September 25, 2017. Soil samples for mineral N and pH were taken on December 8, 2017. Soil samples for chemical and texture analyses were taken in 2012 from 0-20 cm depth. Soil samples for physical analyses were taken in 2014 from three depths. (*) - Bulk density and pore volume % values in this table from the sampling location closest to each plot and depth.

Treatment	Plot #	Sep 25 DM yield g m^{-2}	Sep 25 clover share of DM	Dec 8 mg NH_4^+ N kg^{-1} dry soil, 0-10 cm	Dec 8 mg NO_3^- N kg^{-1} dry soil, 0-10 cm	Dec 8 pH 10mM $CaCl_2$, 0-10 cm	Freeze-Thaw Cum. Flux mg N_2O-N m^{-2}	"Expected" Freeze-thaw flux in mixture from clover share	Spring Thaw Cum. Flux mg N_2O-N m^{-2}	"Expected" Spring thaw flux in mixture from clover share	Post Spring Thaw Cum. Flux mg N_2O-N m^{-2}	"Expected" Post spring thaw flux in mixture from clover share
G-con	214	551		1.01	0.09	5.49	-0.63		3.27		89.95	
G-con	518	528		1.08	0.49	5.62	0.71		14.36		83.53	
G-con	606	689		1.64	0.13	4.90	1.70		3.35		14.87	
G-con	622	513		1.34	0.15	4.68	2.20		10.82		151.77	
G-dol	112	526		1.61	0.14	6.10	0.36		5.34		32.60	
G-dol	424	552		2.09	0.29	5.78	-1.87		6.50		49.39	
G-dol	508	628		1.84	0.29	6.15	2.47		14.21		23.40	
G-dol	628	563		**5.79**	**4.75**	6.12	0.97		2.64		**200.98**	
M-con	315	458	0.55	2.35	1.32	5.19	23.19	46.15	12.47	37.18	11.37	49.14
M-con	321	521	0.49	1.85	1.86	5.18	3.79	47.32	8.08	38.81	11.89	58.61
M-con	331	595	0.44	2.72	1.11	5.08	8.98	48.24	42.72	40.44	29.60	70.09
M-con	521	467	0.48	2.47	1.59	5.10	18.87	41.88	12.16	34.44	17.04	52.93
M-dol	107	466	0.22	3.43	1.35	6.09	6.71	33.20	6.26	17.61	7.98	53.58
M-dol	127	589	0.43	2.85	3.13	6.15	10.43	81.65	12.97	36.59	35.88	55.69
M-dol	329	462	0.52	2.81	1.61	6.38	29.90	77.36	57.78	33.51	25.62	39.70
M-dol	527	476	0.35	**12.71**	**7.73**	5.63	-1.26	54.68	12.58	25.48	**100.93**	48.40
R-con	401	324		1.81	1.39	5.20	45.42		73.12		42.95	
R-con	415	363		1.55	2.77	5.10	60.97		13.79		7.17	
R-con	505	299		1.81	1.82	5.48	58.91		69.05		23.97	
R-con	617	378		2.00	3.99	5.12	86.25		19.56		8.30	
R-dol	211	369		2.40	3.60	6.30	127.23		7.83		1.45	
R-dol	305	337		3.26	3.92	6.23	59.14		41.94		15.48	
R-dol	323	494		4.37	4.03	5.99	149.64		23.14		24.10	
R-dol	429	358		1.97	5.07	6.16	168.66		115.81		19.96	

Freeze-Thaw time-integrated accum. g N_2O-N * day * 0.48m^{-3}	Deep Snow Soil time-integrated accum. g N_2O-N * day * 0.48m^{-3}	Spring Thaw Soil time-integrated accum. g N_2O-N * day * 0.48m^{-3}	% Total C, 0-20 cm	% Total N, 0-20 cm	% Clay, 0-20 cm	% Silt, 0-20 cm	% Sand, 0.20 cm	BD* g cm^{-3} 0-16 cm	BD* g cm^{-3} 16-32 cm	BD* g cm^{-3} 32-48 cm	Pore vol %* 0-16 cm	Pore vol %* 0-16-32 cm	Pore vol %* 32-48 cm
5 747	36 113	4 479	2.6	0.23	26.9	52.2	21.0	0.99	1.57	1.76	61.3	41.3	36.3
6 064	26 254	11 271	3.2	0.28	30.0	48.9	21.1	1.15	1.58	1.82	56.0	41.5	31.7
			2.3	0.20	18.2	32.5	49.3	1.31	1.83	1.66	47.4	31.6	37.8
9 569	36 511	10 348	3.1	0.31	29.2	48.7	22.1	1.14	1.50	1.57	56.3	44.2	42.8
5 552	16 961	6 451	2.8	0.24	28.2	52.0	19.7	1.15	1.62	1.76	53.5	39.2	36.3
4 759	9 972	4 252	3.0	0.28	29.7	52.6	17.7	1.04	1.62	1.60	59.8	40.5	41.9
			2.6	0.23	20.9	36.0	43.1	1.31	1.83	1.66	47.4	31.6	37.8
6 927	24 682	5 548	3.3	0.31	31.4	52.2	16.4	1.24	1.50	1.65	52.7	44.7	39.8
17 248	628 405	8 453	3.3	0.30	31.5	51.0	17.6	1.18	1.41	1.68	55.2	46.4	37.7
5 843	164 901	14 984	3.3	0.29	30.7	53.1	16.2	1.18	1.68	1.60	55.2	37.5	41.9
			2.6	0.22	26.3	52.4	21.3	1.15	1.60	1.72	52.5	41.1	37.0
17 085	1 652 870	492 674	3.1	0.28	30.0	51.1	18.9	1.23	1.35	1.51	52.2	49.3	43.1
			3.5	0.30	31.6	45.9	22.4	1.13	1.41	1.68	56.0	46.4	37.7
15 793	277 022	178 347	2.5	0.25	19.9	58.2	21.9	1.15	1.45	1.54	56.2	45.5	40.6
12 466	453 994	182 573	2.7	0.24	28.1	54.0	17.9	1.15	1.60	1.72	52.5	41.1	37.0
13 004	574 940	113 297	3.3	0.31	30.7	52.7	16.6	1.19	1.51	1.65	54.0	45.4	39.8
			2.2	0.17	21.8	37.0	41.2	1.05	1.69	1.66	60.4	36.7	37.8
43 581	1 500 226	4 084	3.3	0.29	32.1	50.6	17.3	1.11	1.41	1.68	56.9	46.4	37.7
152 847	3 550 234	981 089	2.5	0.22	19.9	35.4	44.8	1.31	1.83	1.66	47.4	31.6	37.8
138 139	2 317 284	12 621	3.2	0.28	29.5	47.0	23.6	1.15	1.58	1.82	56.0	41.5	31.7
128 400	1 036 484	3 901	2.8	0.24	27.6	50.7	21.7	0.99	1.57	1.76	61.3	41.3	36.3
			2.8	0.27	21.6	35.5	42.9	1.14	1.36	1.78	55.3	47.1	34.7
111 598	1 907 272	124 404	3.2	0.30	29.8	52.8	17.4	0.99	1.68	1.60	61.5	37.5	41.9
61 798	2 436 153	554 353	2.8	0.27	28.6	54.2	17.3	1.12	1.63	1.72	55.7	40.4	37.0

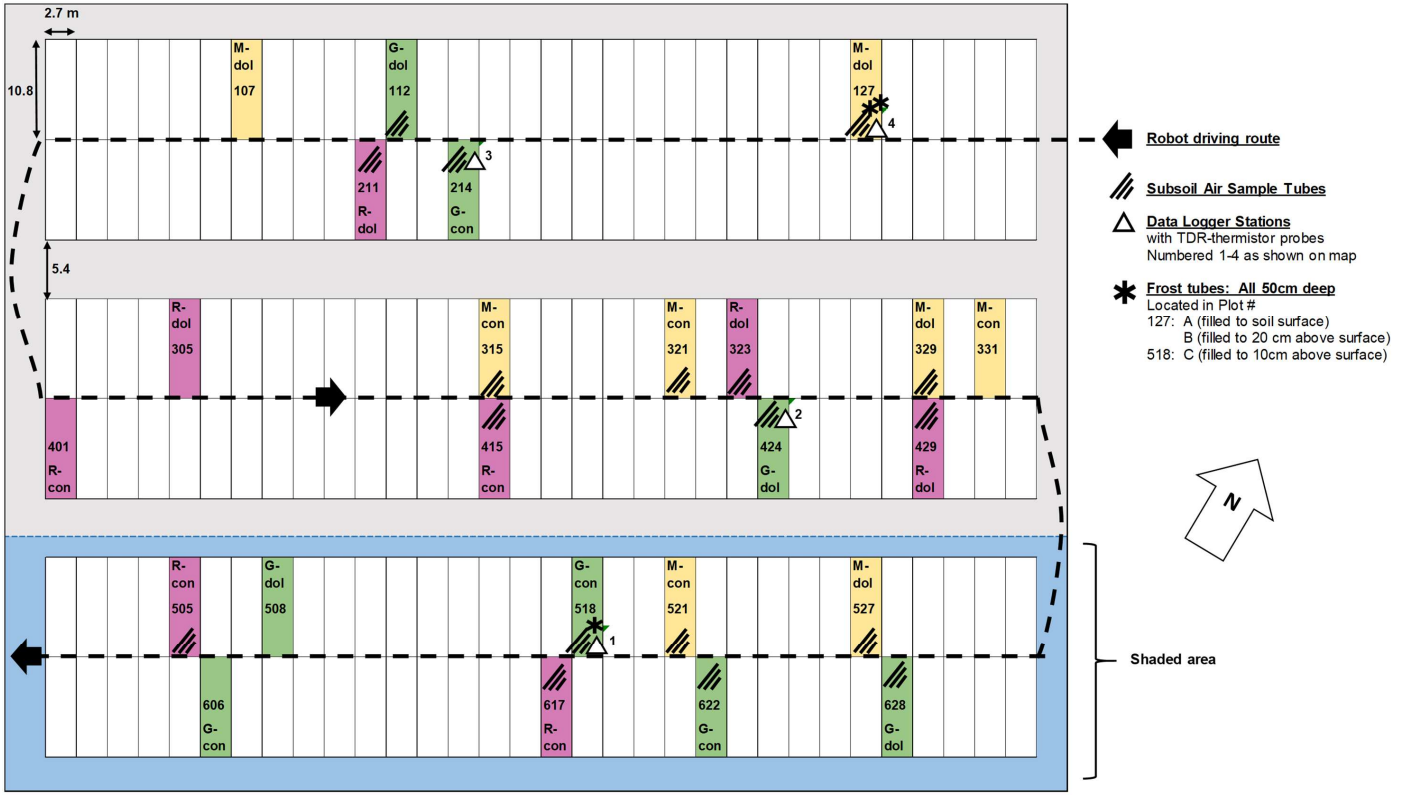


Figure S.1: Map of experimental setup.

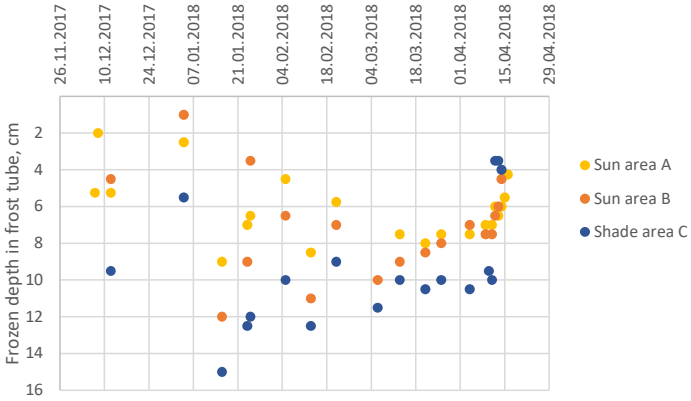


Figure S.2: Depth of soil freezing as estimated by frost tubes. Two frost tubes (A and B) were placed in the sunny area of the field. A third frost tube (C) was placed in the shaded area (see Figure S.1 – Map of experiment). All frost tubes were unfrozen from April 16 onward.

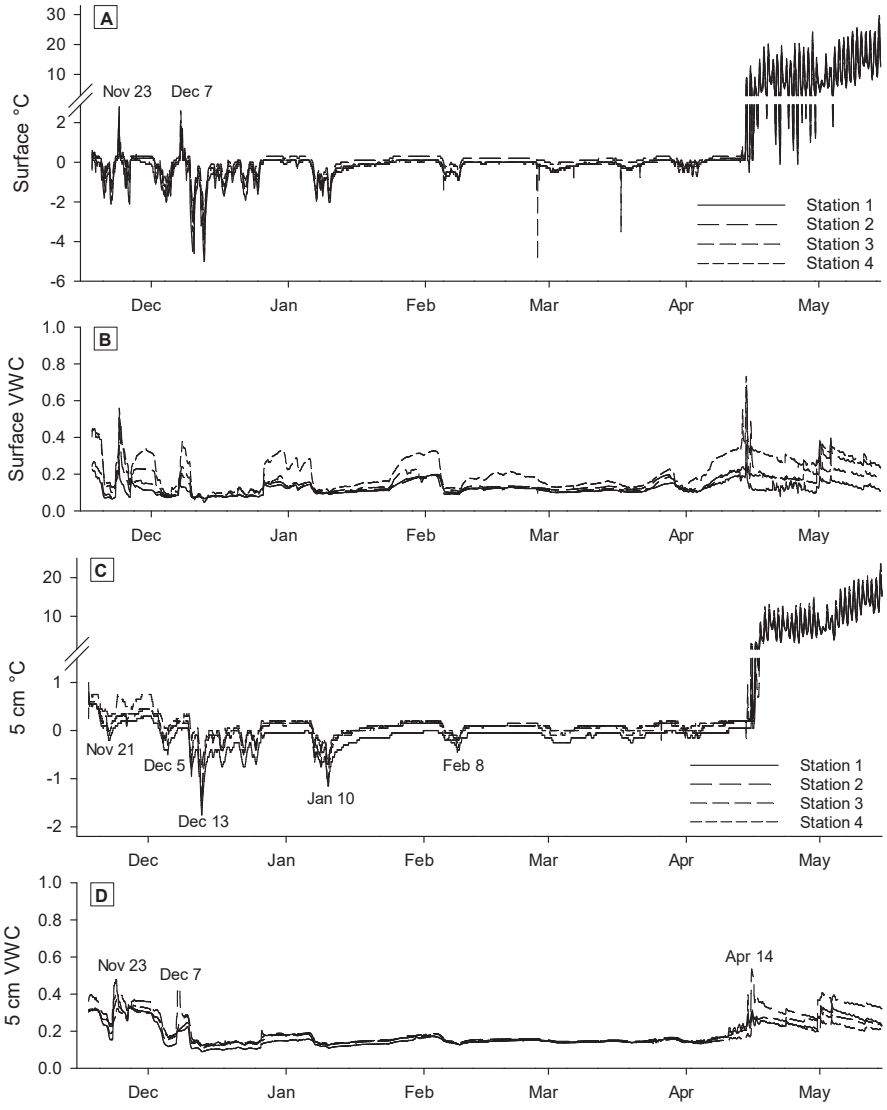


Figure S.3 (1 of 2): Hourly average temperature °C and soil VWC measurements from TDR-thermistor probes at four stations within the field (See Figure S.1 Map of experiment), A-B) placed just below the soil surface; C-D) average of two probes per station placed at 5 cm depth

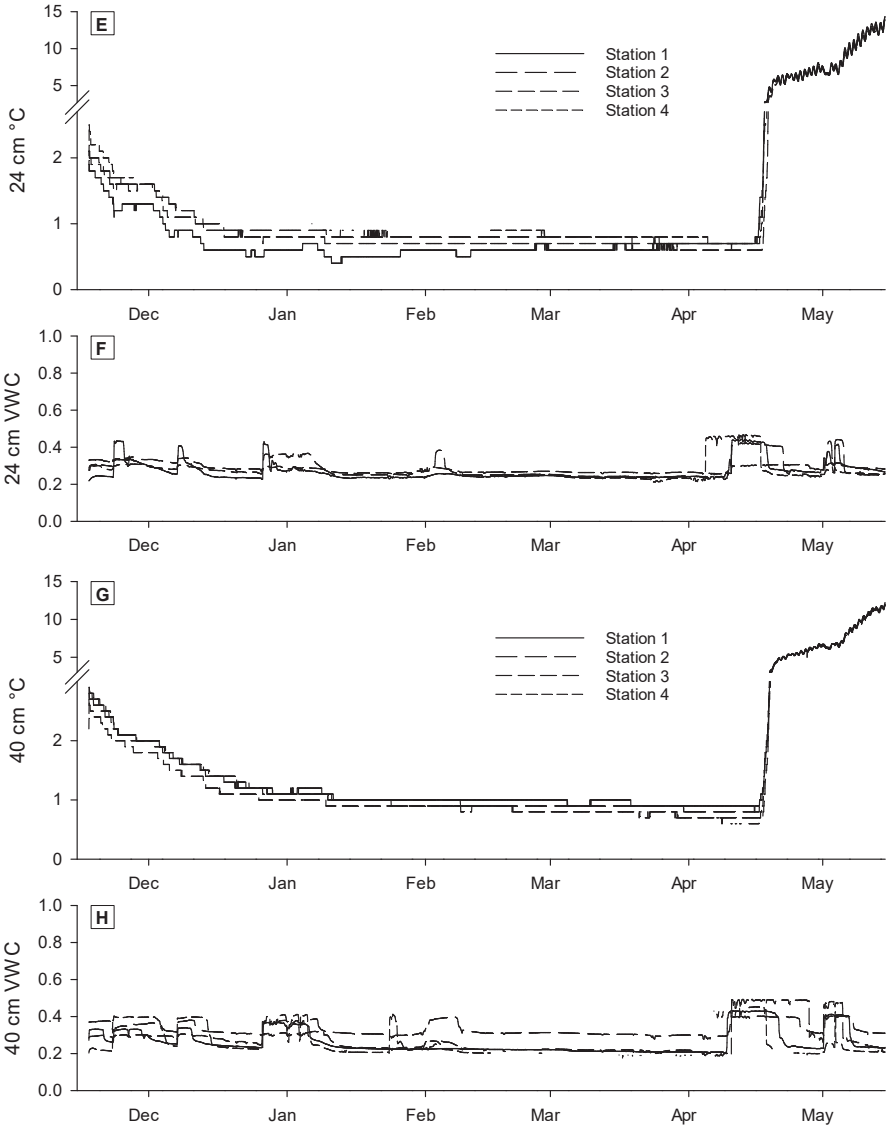


Figure S.3 (2 of 2): Hourly average temperature °C and soil VWC measurements from TDR-thermistor probes at four stations within the field (See Figure S.1 Map of experiment), E-F) placed at 24 cm depth; G-H) placed at 40 cm depth

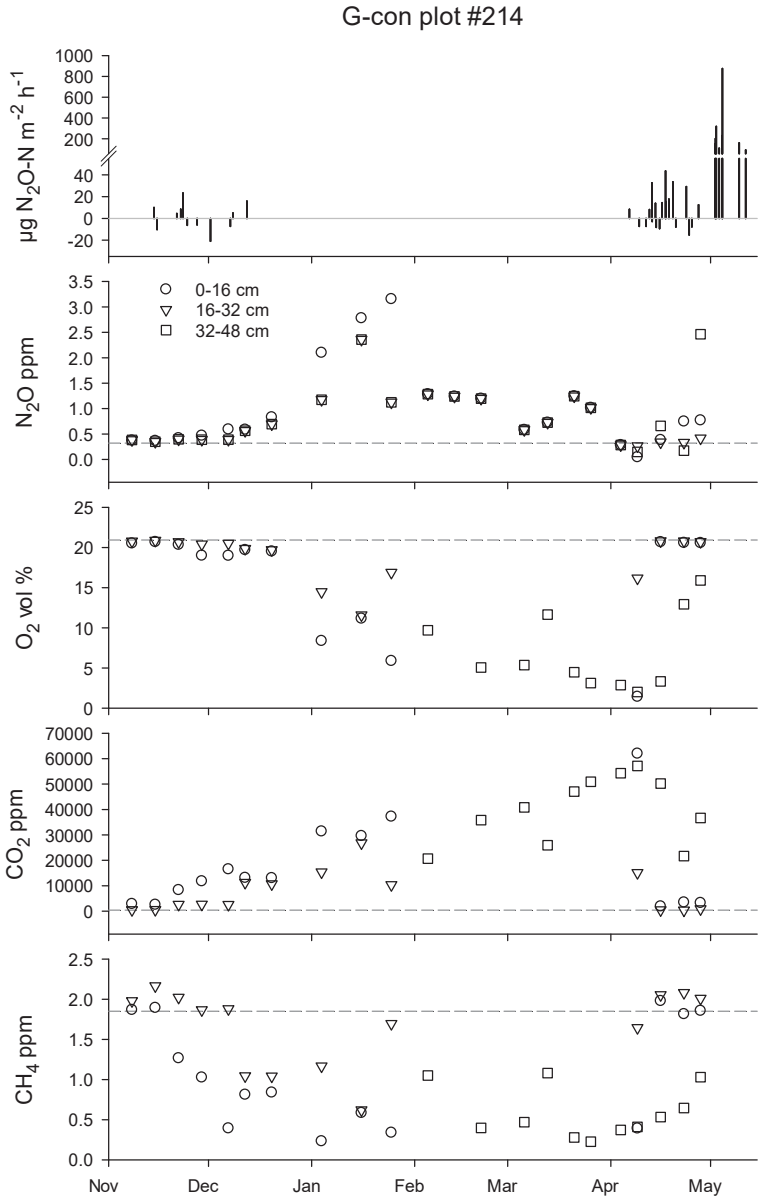


Figure S.4 (1 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

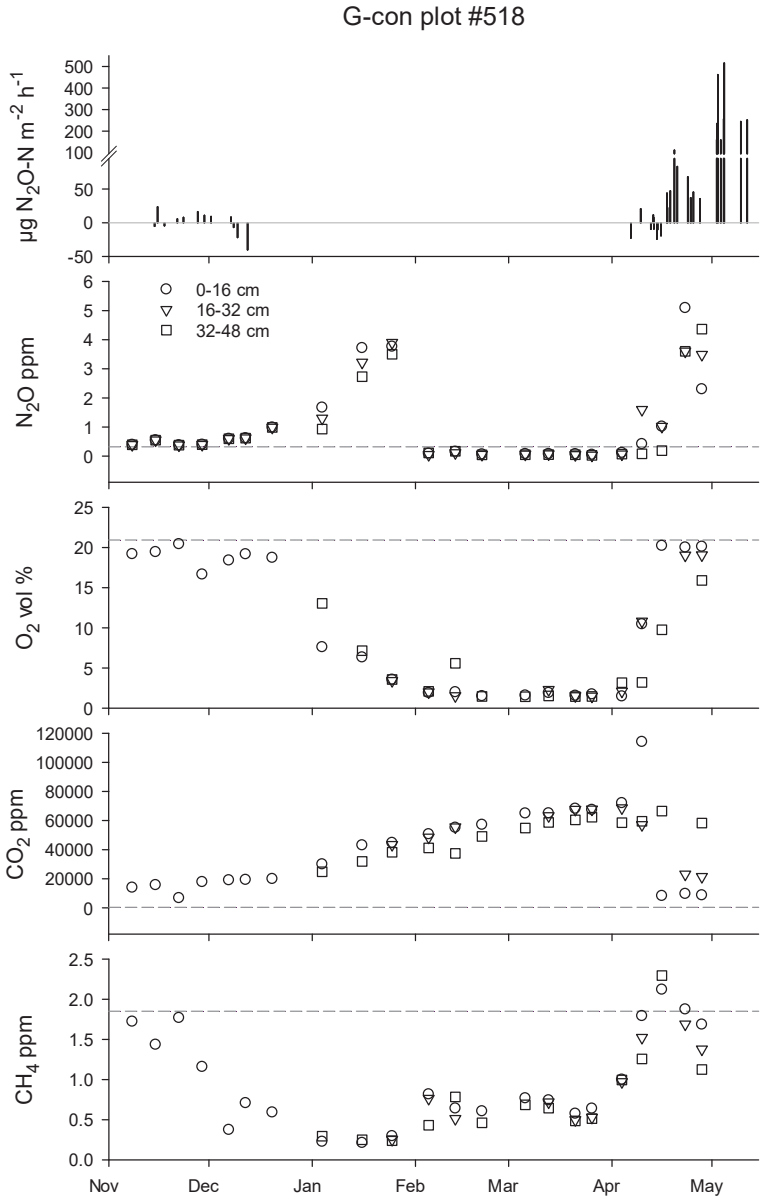


Figure S.4 (2 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

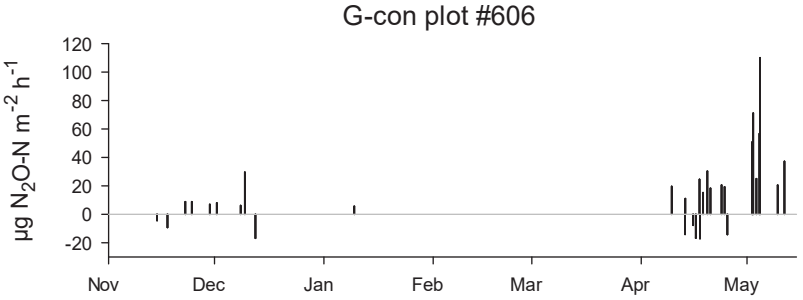


Figure S.4 (3 of 24): For individual subplot: Measured flux µg N_2O-N m^{-2} hr^{-1}.

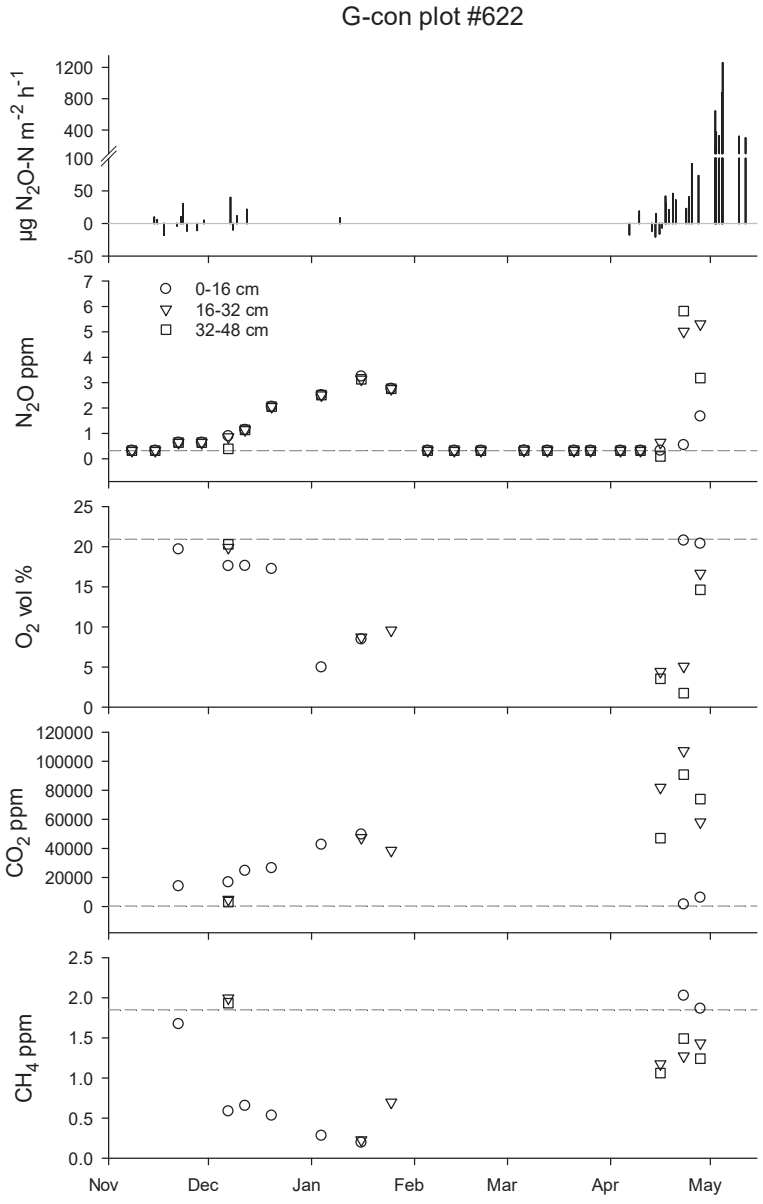


Figure S.4 (4 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

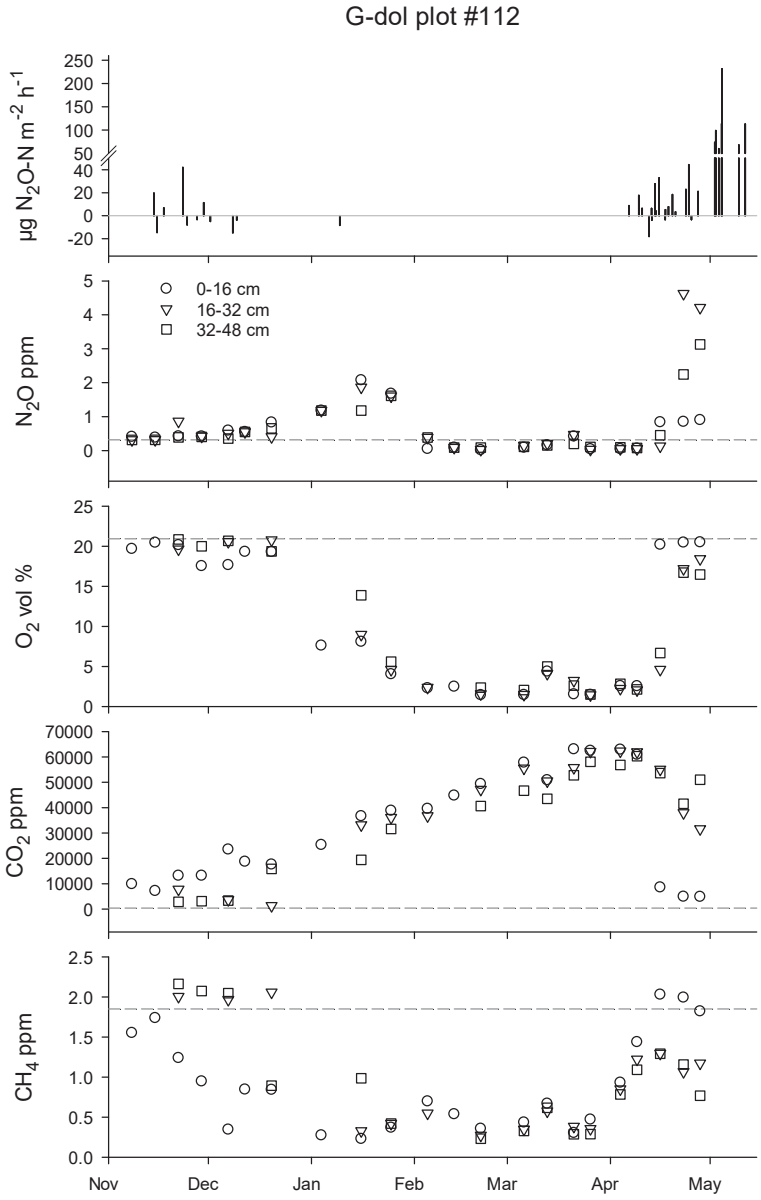


Figure S.4 (5 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

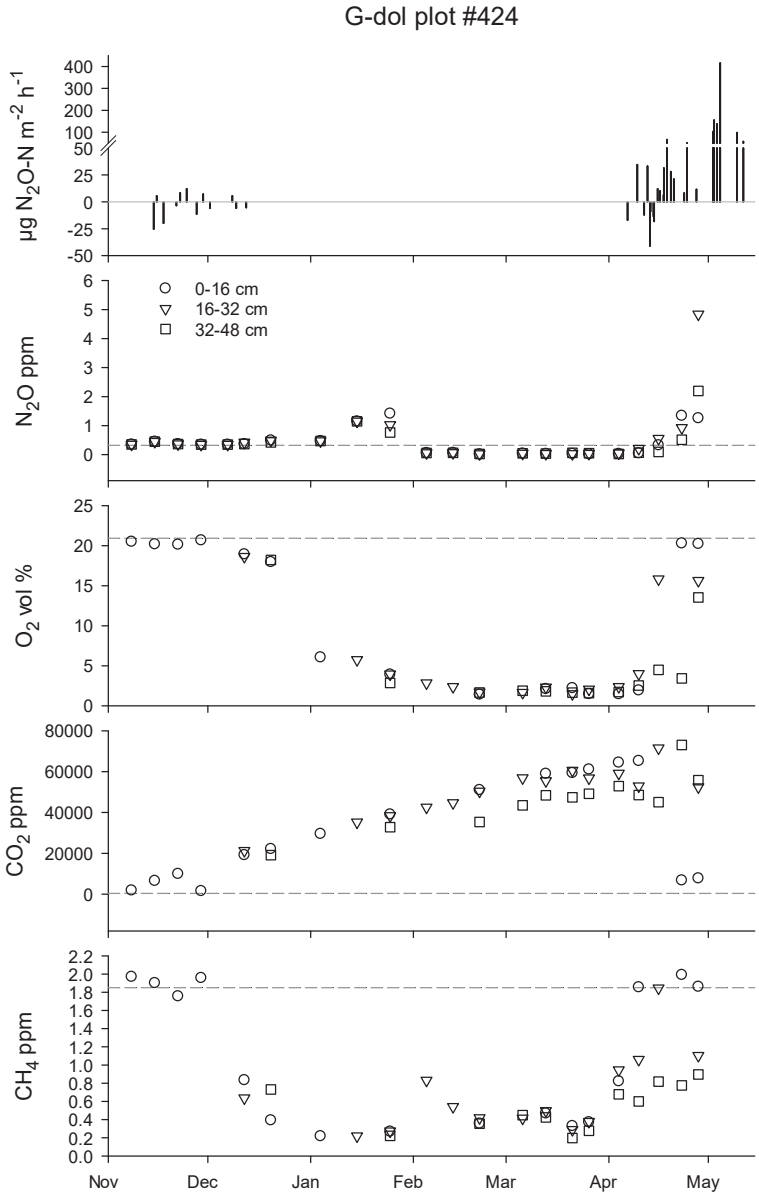


Figure S.4 (6 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

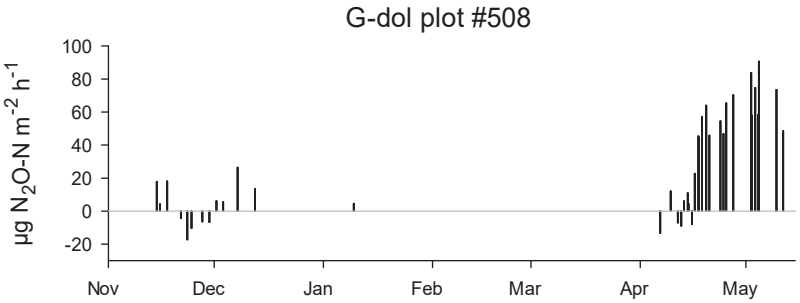


Figure S.4 (7 of 24): For individual subplot: Measured flux µg N_2O-N m^{-2} hr^{-1}.

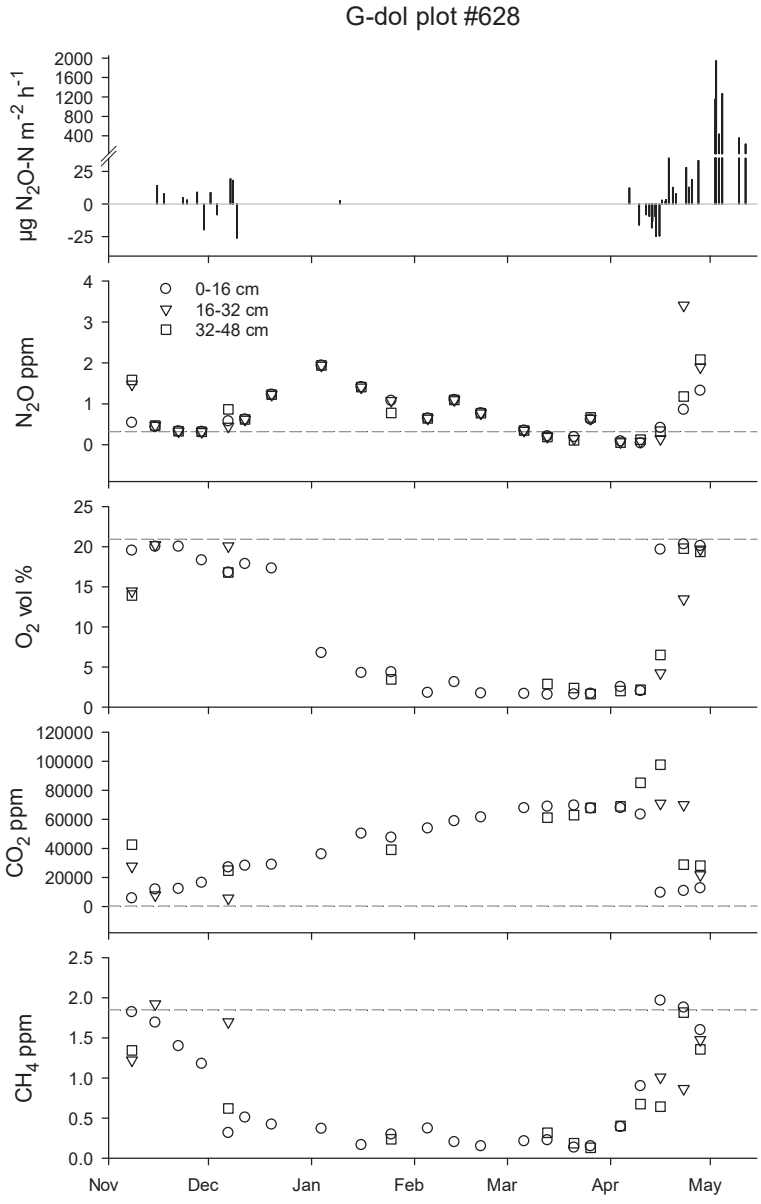


Figure S.4 (8 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

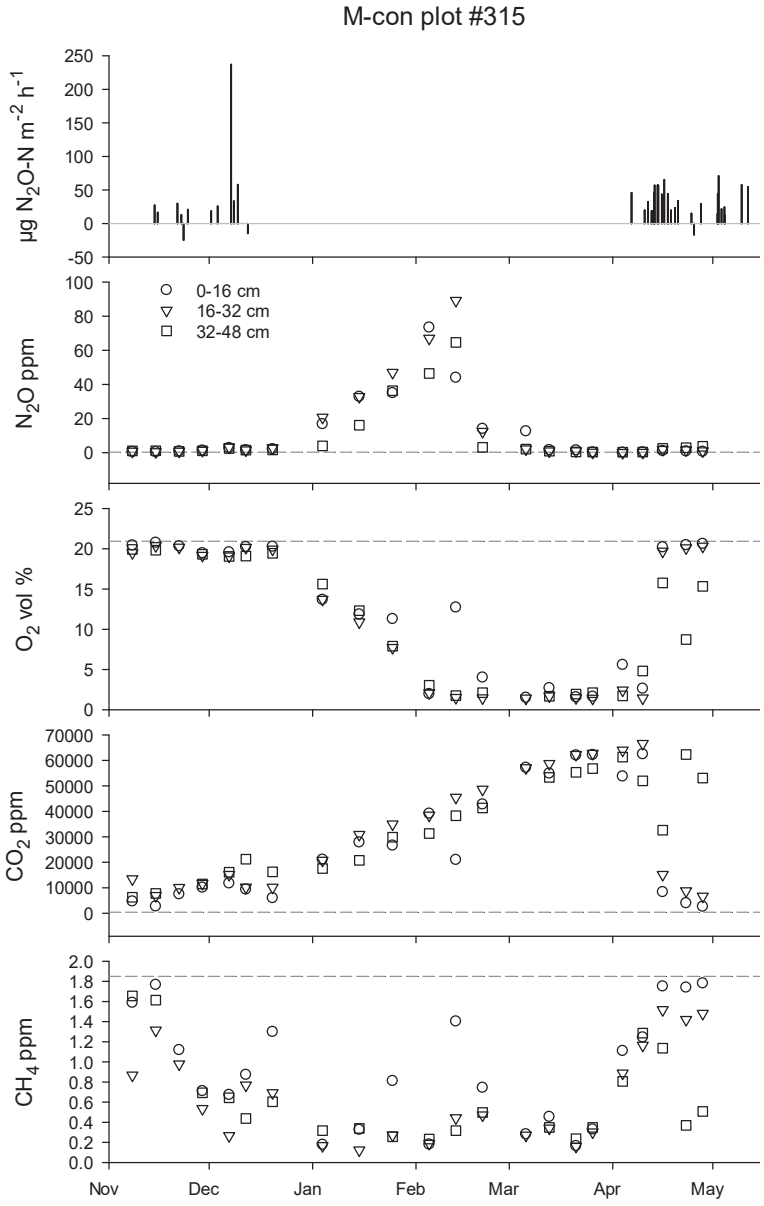


Figure S.4 (9 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

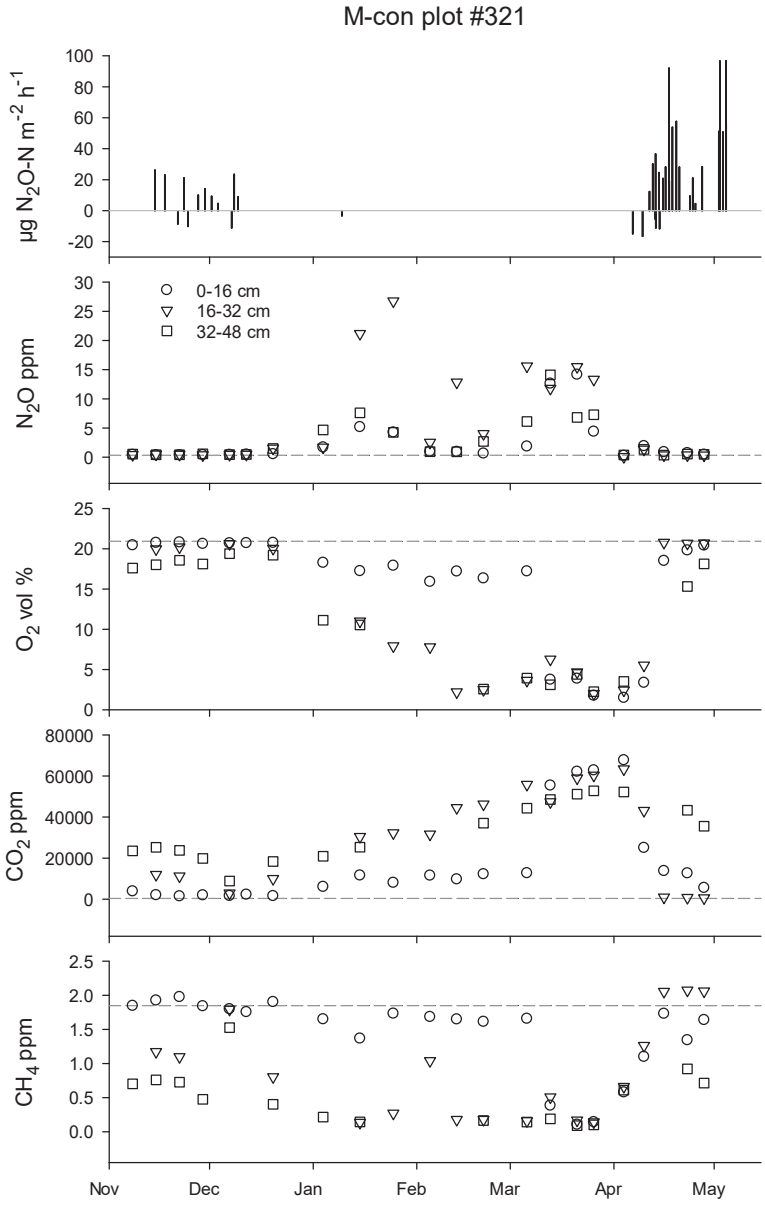


Figure S.4 (10 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

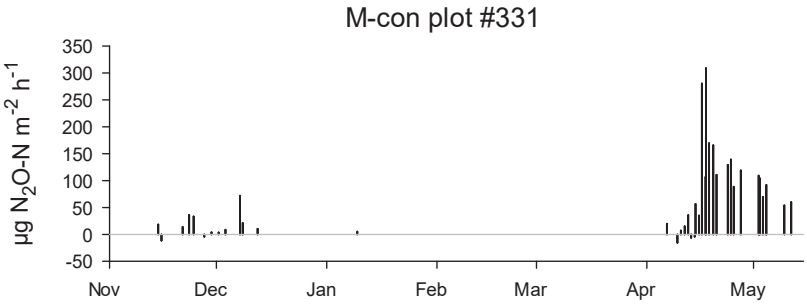


Figure S.4 (11 of 24): For individual subplot: Measured flux µg N_2O-N m^{-2} hr^{-1}.

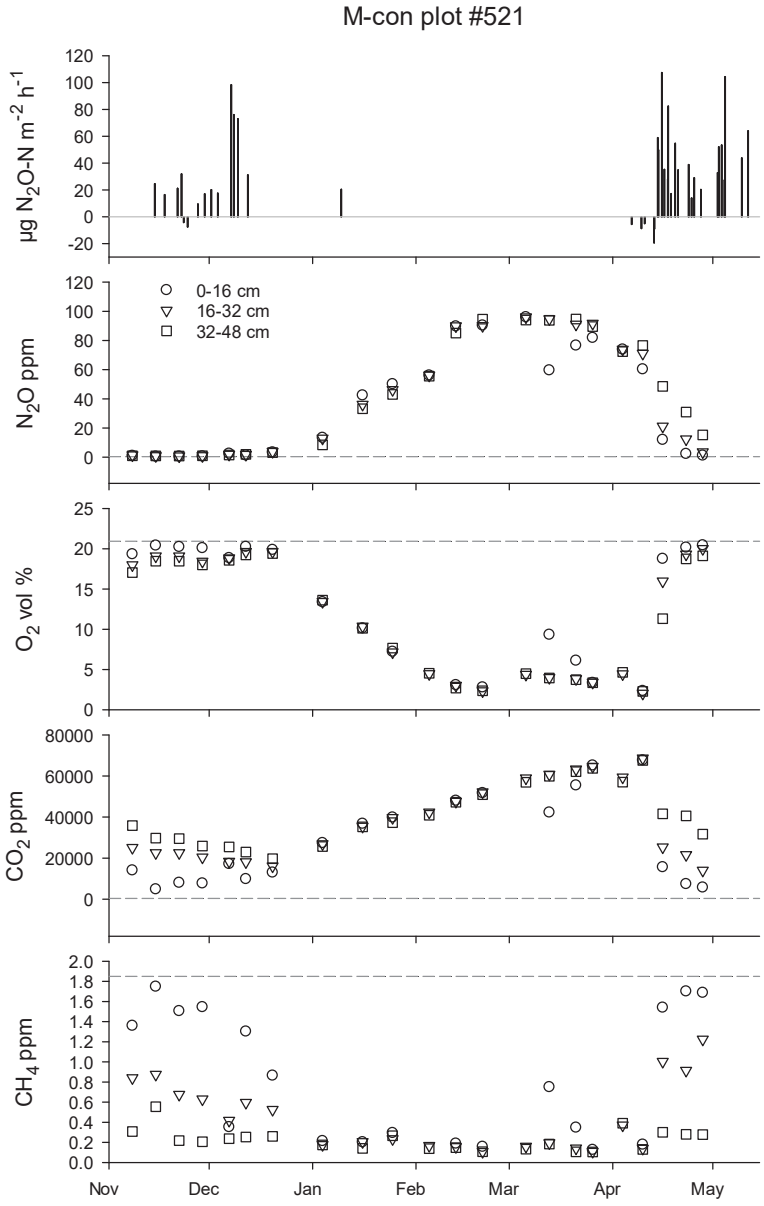


Figure S.4 (12 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

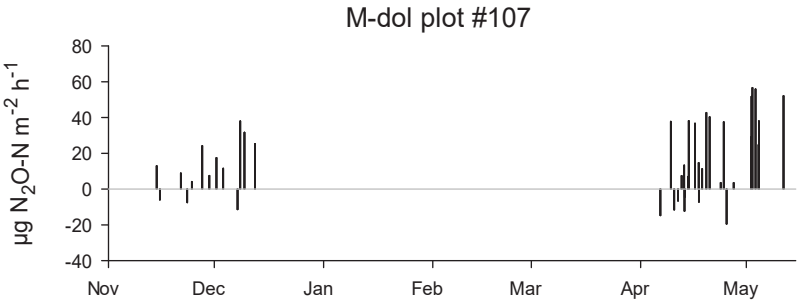


Figure S.4 (13 of 24): For individual subplot: Measured flux μg N_2O-N m^{-2} hr^{-1}.

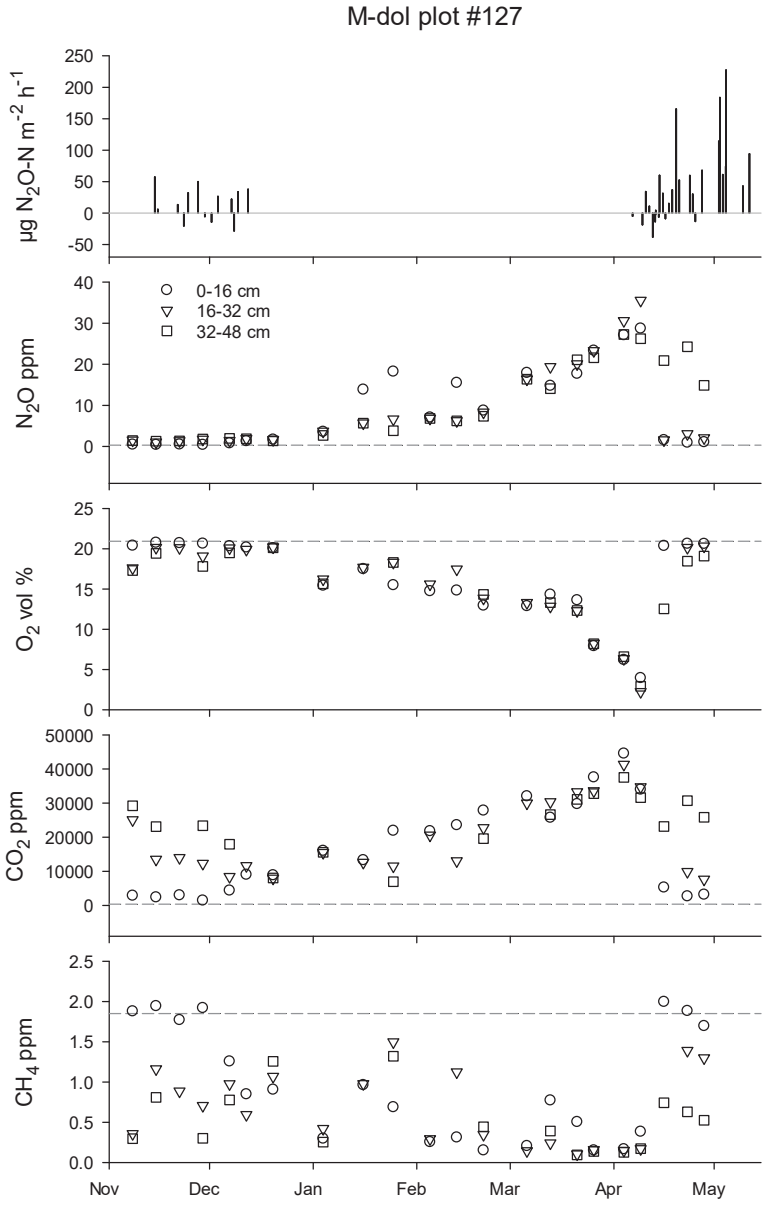


Figure S.4 (14 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

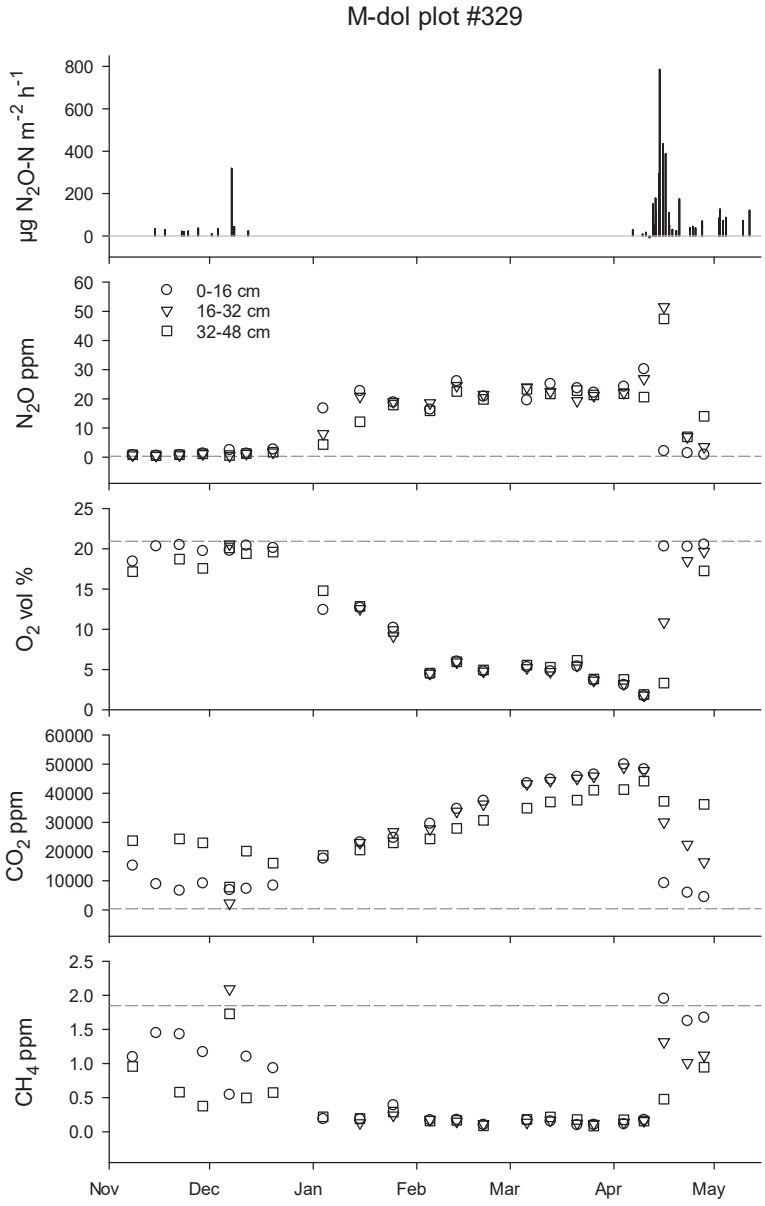


Figure S.4 (15 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

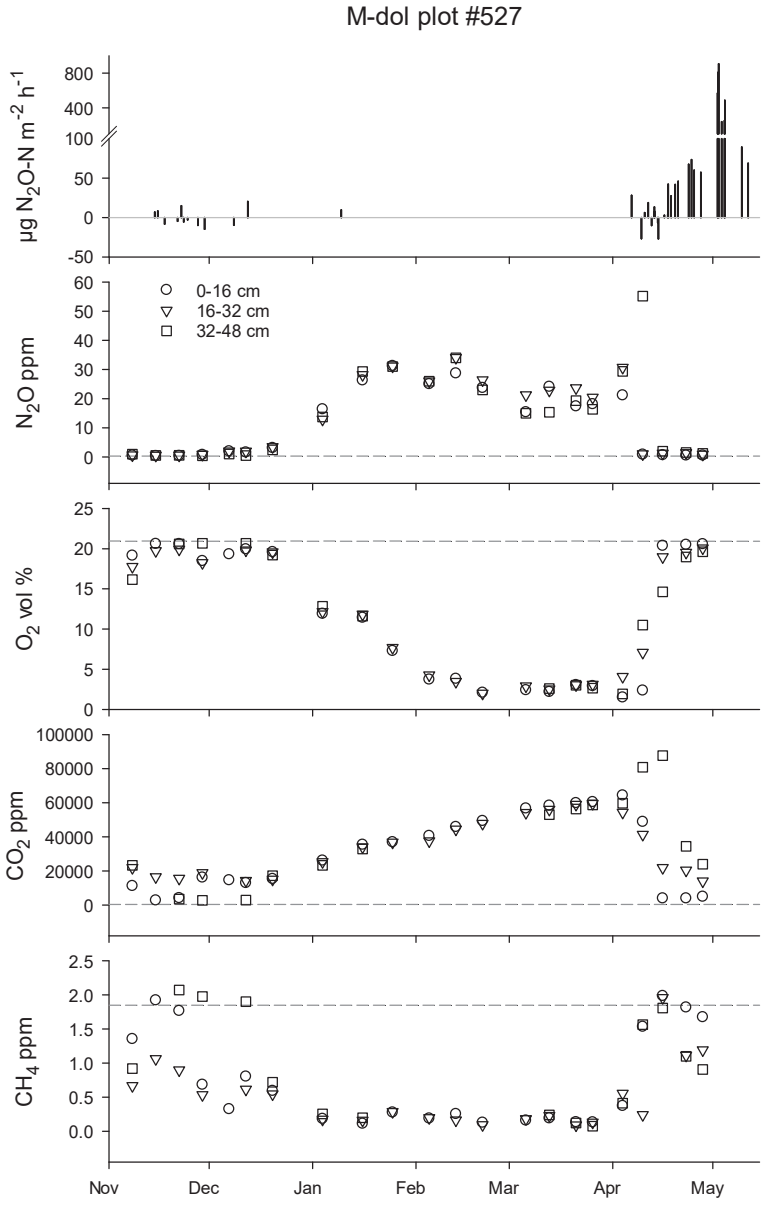


Figure S.4 (16 of 24): For individual subplot, from top: Measured flux μg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

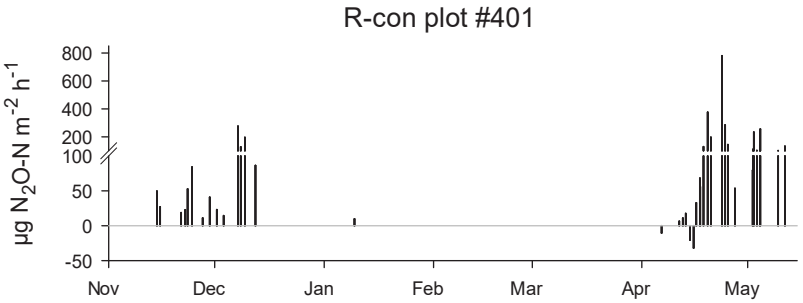


Figure S.4 (17 of 24): For individual subplot: Measured flux µg N_2O-N m^{-2} hr^{-1}.

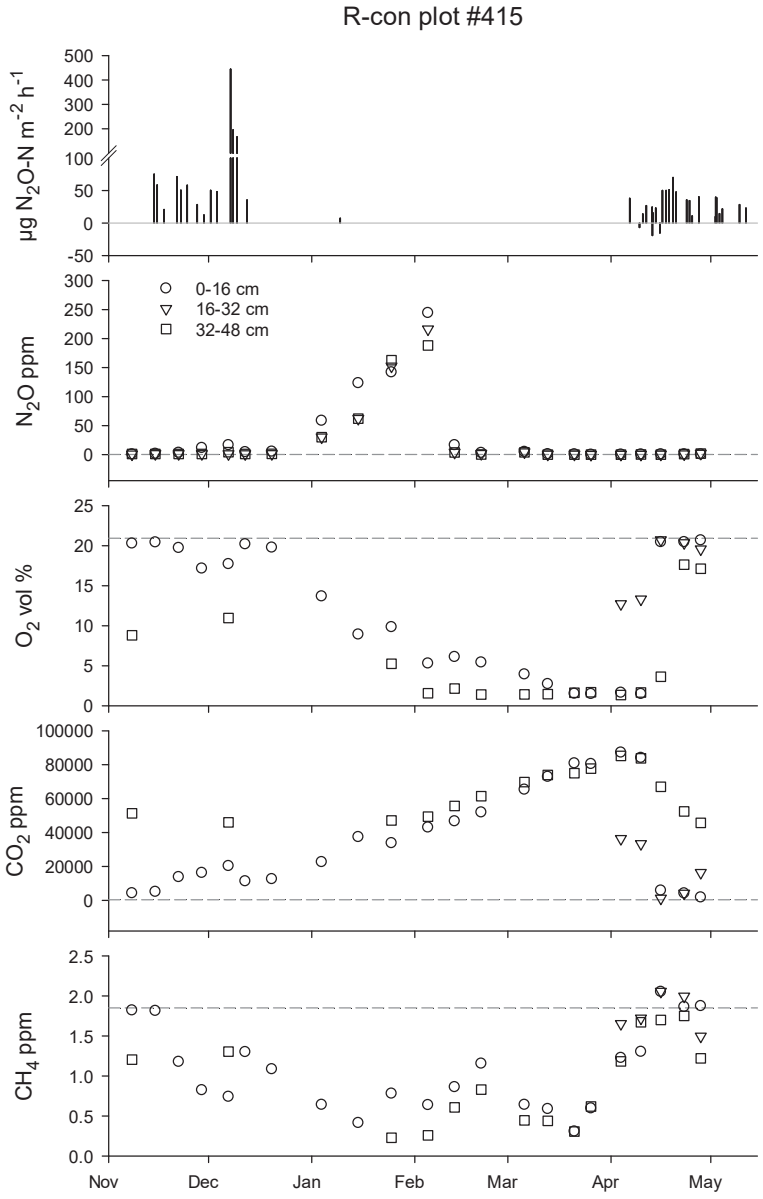


Figure S.4 (18 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

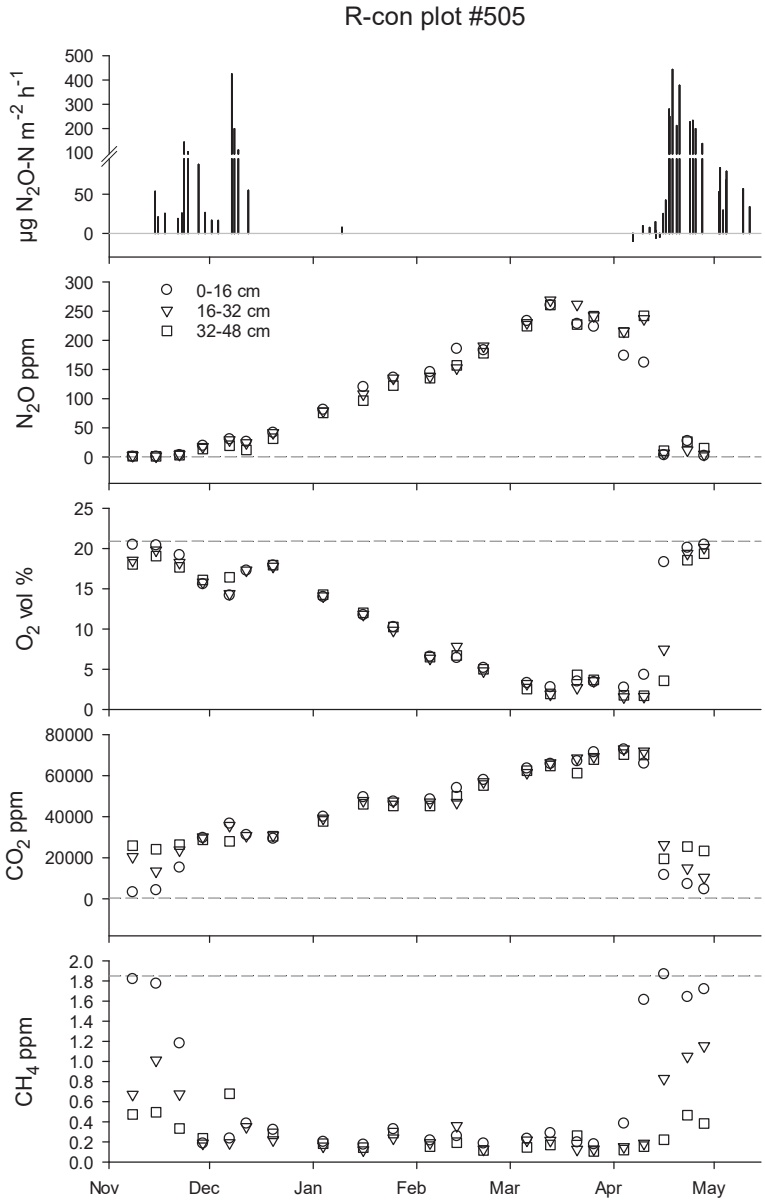


Figure S.4 (19 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

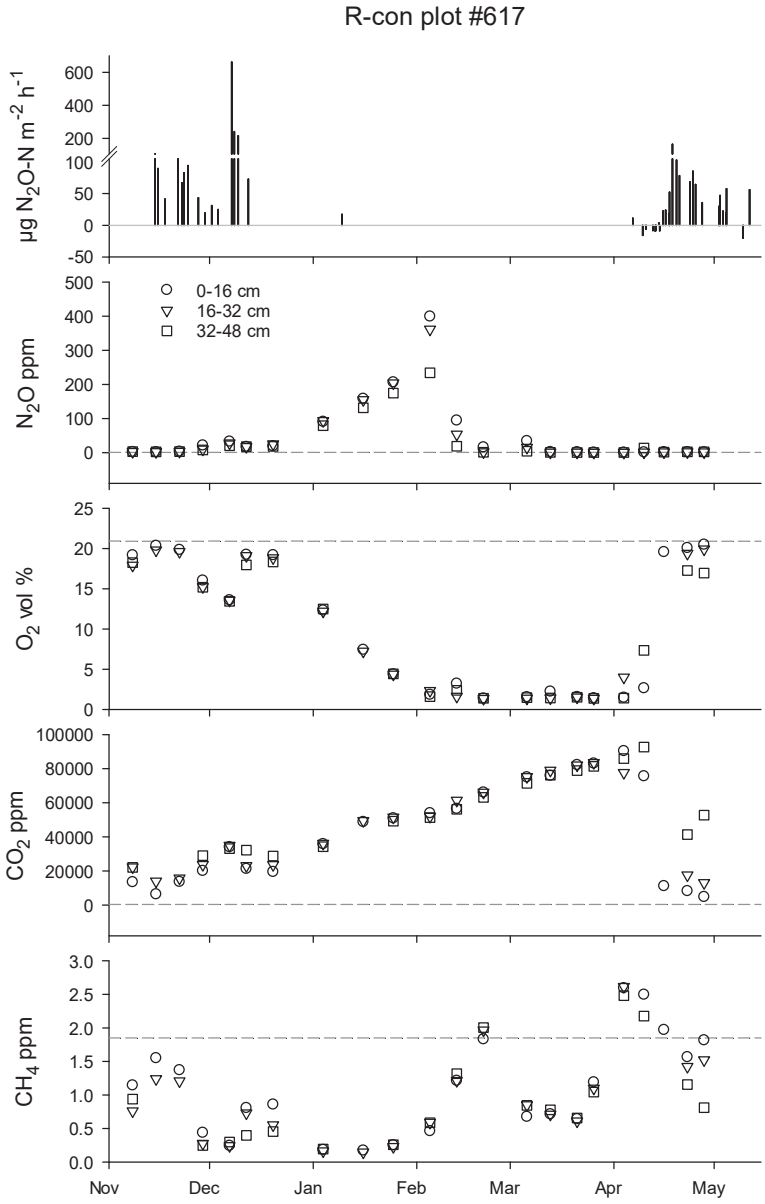


Figure S.4 (20 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

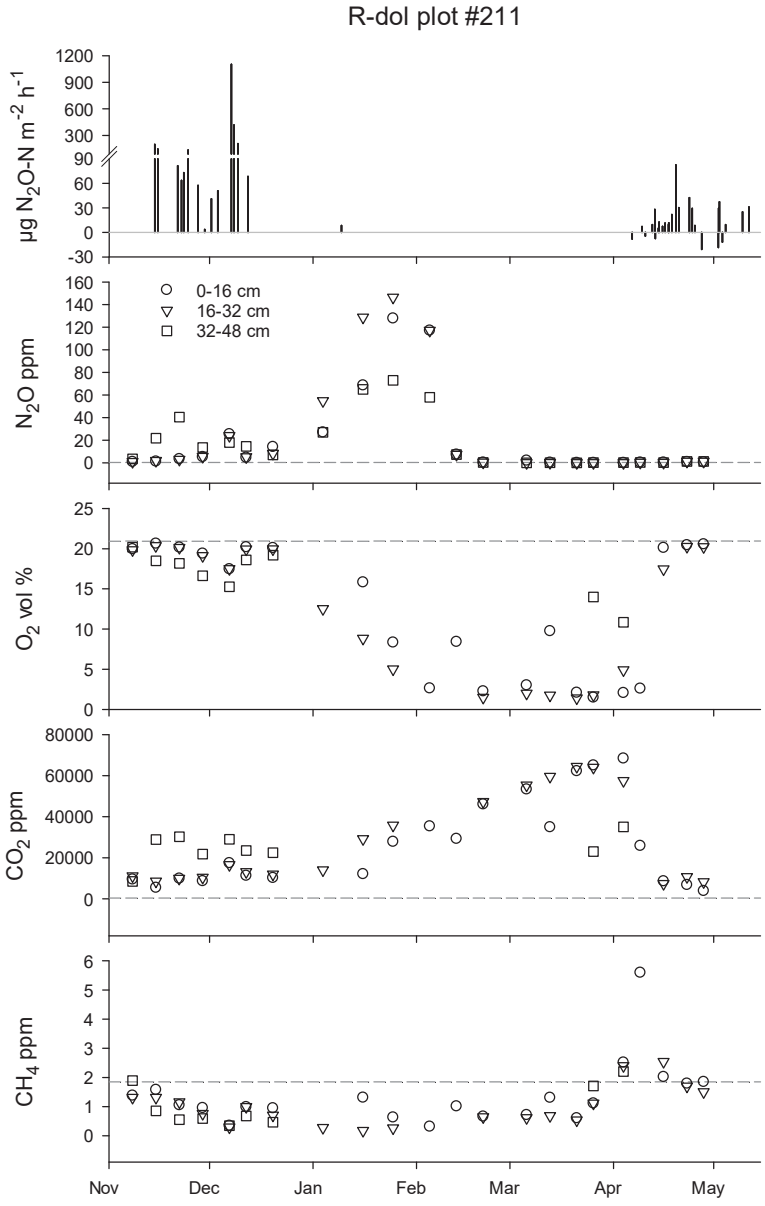


Figure S.4 (21 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

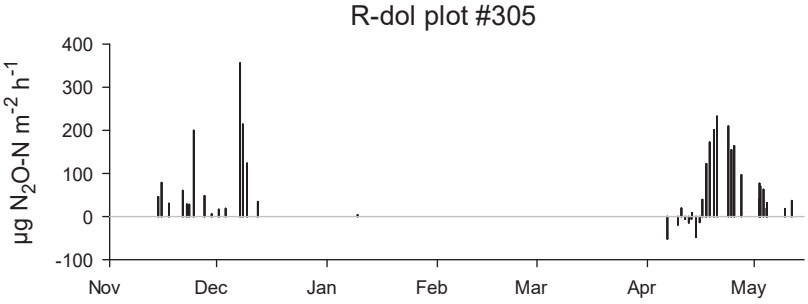


Figure S.4 (22 of 24): For individual subplot: Measured flux µg N_2O-N m^{-2} hr^{-1}.

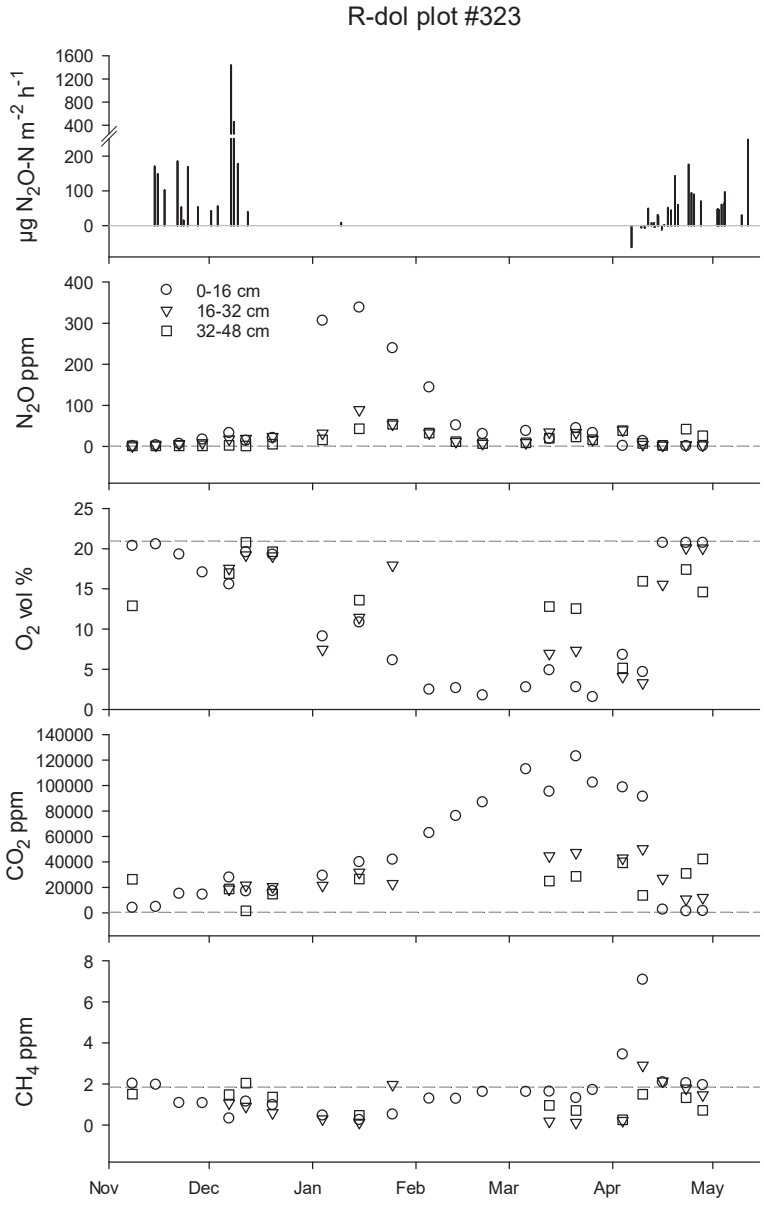


Figure S.4 (23 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

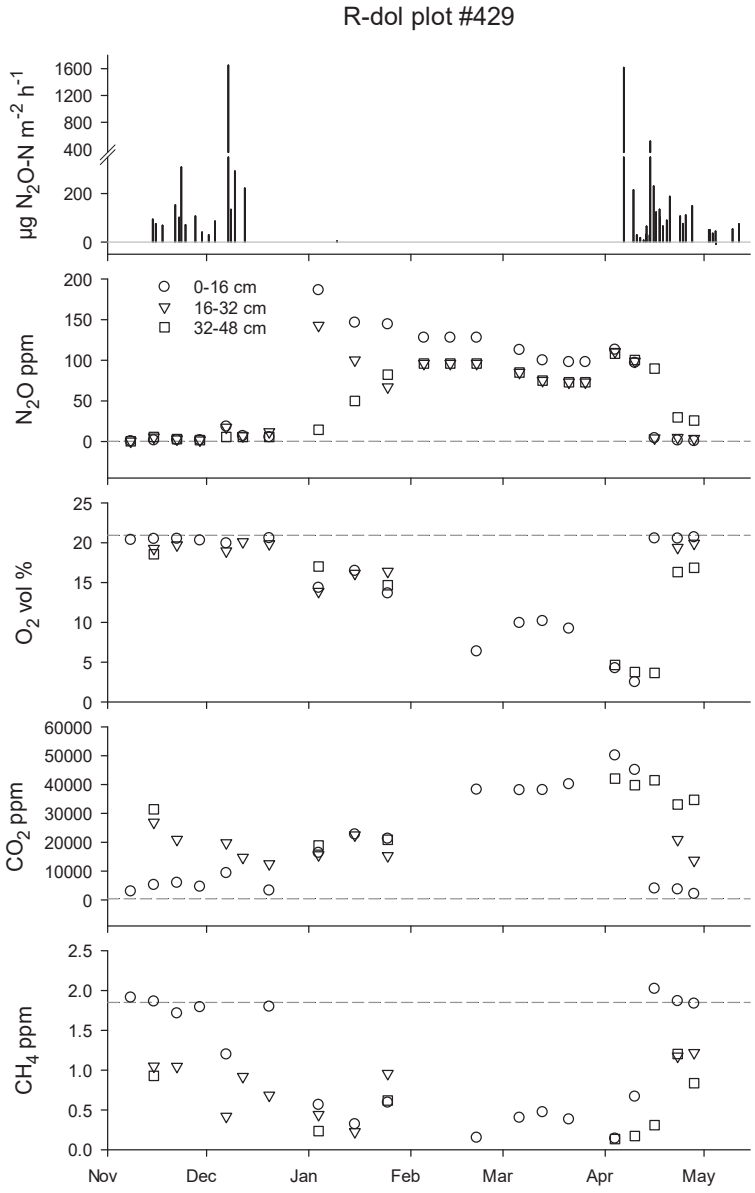


Figure S.4 (24 of 24): For individual subplot, from top: Measured flux µg N_2O-N m^{-2} hr^{-1}. Measured soil air concentrations at individual depths: N_2O, O_2, CO_2, and CH_4. Soil air sampling was not possible where marks are missing for O_2, CO_2, and CH_4; at these times N_2O concentration was interpolated. See Methods 2.7.1: Accumulation of N_2O in soil. Dashed gray lines show ambient atmospheric levels of N_2O (0.32 ppm), O_2 (20.95%), CO_2 (400 ppm), and CH_4 (1.85 ppm).

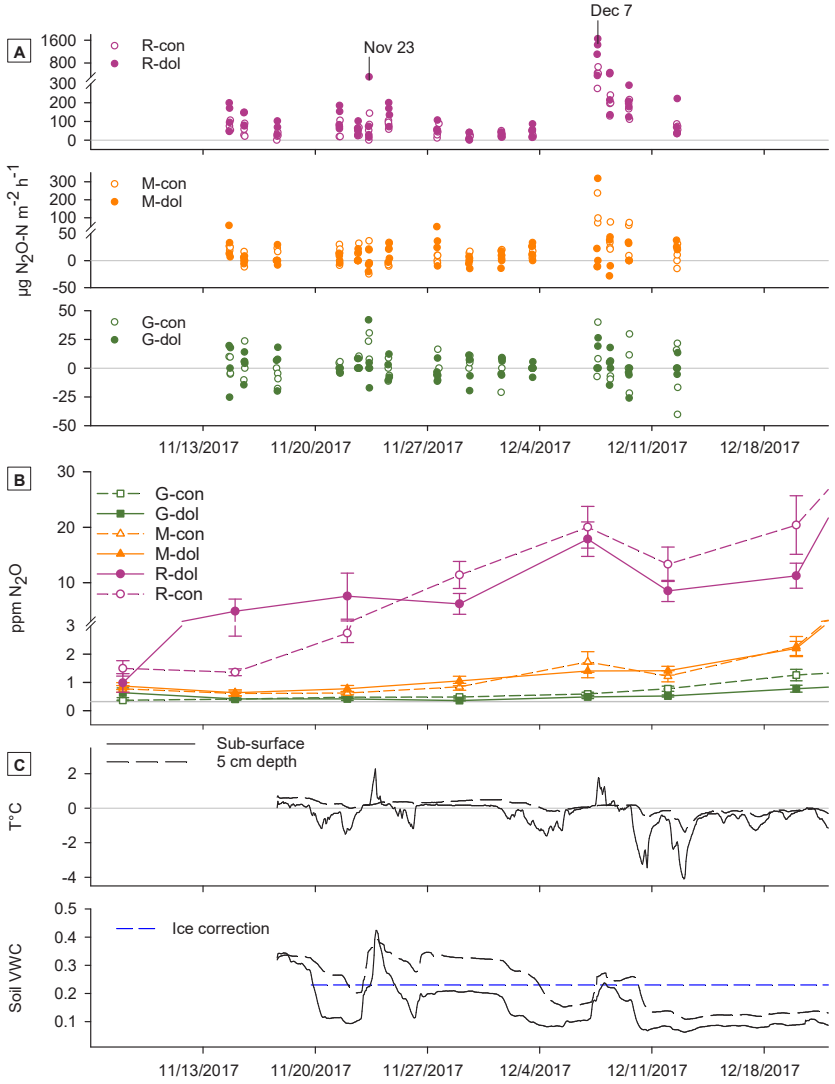


Figure S.5: Detail of autumn freeze-thaw period. A) Individual surface flux measurements in µg N_2O-N m^{-2} h^{-1} for each treatment (note difference in scales). All replicates shown. B) Concentration of N_2O in ppm measured in soil air samples for each treatment. Average of 3 depths. Error bars are SE of 3 replicates for N_2O, where missing values were interpolated (see Methods, 2.7.1 Accumulation of N_2O in soil). Individual depth and plot measurements are available in Supplementary Figure S 3. Gray line indicates the ambient atmospheric level of N_2O (0.32 ppm). C) Soil temperature °C and soil VWC just below the soil surface and at 5 cm depth (mean of 4 logging stations). Dashed line in SVWC chart shows assumed volume including frozen water (See Methods, 2.7.1 Accumulation of N_2O in soil).

ISBN: 978-82-575-2035-9
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

116345 / ANDVORDGRAFISK.NO