# Urea-induced Nitrous Oxide Emission in Multi-species Swards: Effect of Species and Density

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## Abstract

Grazed grasslands have been identified as an important source of anthropogenic nitrous oxide ($N_2O$) emissions. The loss of soil N as $N_2O$ is a critical factor for managing sustainable agroecosystems, not only with respect to the contribution of $N_2O$ to global warming by radiative forcing, but also for its effect on stratospheric ozone depletion. An experiment was set up on the experimental farm Østervoll, SE Norway, to investigate the effect of clover density in multi species swards (MSS) on $N_2O$ emissions from urine patches. The species used in the MMS were ryegrass, tall fescue, red clover and white clover. Clover densities ranged from 0-100% in mixed stands with grass yielding all together nine different treatments. We used artificial urine (50 g N $m^{-2}$) to simulate urine deposition and measured $N_2O$ flux using static chambers. Gas samples were analyzed by gas chromatography and soil samples were analyzed for $NH_4^+$ and $NO_3^-$. Harvest took place on the 13$^{th}$ of September and the plant samples were analyzed for N yield and clover percentage. The data obtained were analyzed using one way ANOVA. The results showed no significant differences in cumulative $N_2O$ emission in the period from urea application to ley harvest between grass treatments and grass dominated mixtures, however high emissions were associated with clover monocultures. The high standard error within replicates of the same treatments suggested an effect of topography, resulting in lower emissions in plots situated on a slope, presumably because of nitrogen leaching. When scaled for N-yield, cumulative $N_2O$ emissions tended to be higher for treatments with high clover percentage. In conclusion, clover percentage and species distribution had little effect on urine-associated $N_2O$ emissions. This warrants that there might be tradeoff between increasing N uptake by companion grass and N yield-scaled $N_2O$ emissions in grazed multispecies pastures.



Key words: Clover density, Nitrous oxide, grasslands, urine patches

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# 1. Introduction

## 1.1 Nitrous oxide: greenhouse gas emission and N cycle

**" Most of the global average warming over the past 50 years is very likely due to anthropogenic greenhouse gas (GHG) increases and it is likely that there is a discernible human induced warming averaged over each continent (except Antarctica)" (Intergovernmental Panel for Climate Change, IPCC AR4 synthesis report, 2007)**

Climate change poses a serious threat to all forms of life, starting from loss of biodiversity and natural habitats of a wide range of species, to undermining food production, and increasing crop failures. The increase in the concentration of greenhouse gases in the atmosphere enhances the natural radiative forcing that affects the earth surface temperature. The concentrations of the greenhouse gases carbon dioxide ($CO_2$), methane ($CH_4$) and nitrous oxide ($N_2O$) have increased strongly over those recorded throughout the last 650,000 years (IPCC WGI, 2007). $CO_2$ has increased due to fossil fuel use and land use change, the increase in $CH_4$ is attributed to fossil fuel use, agricultural activities (ruminants) and possibly permafrost melting, while the increase in $N_2O$ concentration is primarily attributed to the ever rising use of reactive nitrogen (N) by agriculture (IPCC, 2007a). However, the magnitude by which anthropogenic GHG emissions contribute to increasing the earth surface temperature is still uncertain (Fang et al., 2011). The concentration of nitrous oxide in the atmosphere has increased globally from its pre-industrial value of 270 ppb to 319 ppb in 2005 (IPCC, 2007a). $N_2O$ has a global warming potential of 298 times as much as that of carbon dioxide (ibid.) The atmospheric $N_2O$ abundance of 314 ppb in 1998 results in an additional radiative forcing (RF) of +0.15 ± 0.02 W $m^{-2}$. The primary driver for the industrial era increase of $N_2O$ was concluded to be enhanced microbial production in expanding and fertilized agricultural lands (IPCC, 2007a).

Nitrous oxide has received particular attention in agro-ecological research, since $N_2O$ emissions from agricultural soils account for 70% of atmospheric $N_2O$ (Mosier, Kroeze et al. 1998). In agroecosystems, anthropogenic emissions of $N_2O$ result from a disruption of the N cycle due to the excessive use of artificial N fertilizers. Thus, they are a form of loss of reactive nitrogen from soil. Early studies showed that $N_2O$ contributes to the photochemical destruction of ozone once it reaches the stratosphere (Crutzen, 1981) with an ozone depleting potential (ODP) comparable to that of Chlorofluorocarbons (CFCs), estimated to be one sixtieth of the ODP of CFC's (Ravishankara, et al. 2009).

Most anthropogenic $N_2O$ emissions are caused by microbial nitrogen transformations in soil and manure. Direct sources of $N_2O$ emission include artificial fertilizers, animal excreta, and cultivation of organic soils and mineralization of N-rich crop residues (Smith, et al. 2000). Indirect emissions occur from leached nitrate ($NO_3^-$), short-range transported and deposited $NH_3$ or from nitrogen oxides ($NO_x$) elsewhere (Mosier 2001). An estimated 5% of $N_2O$ in the atmosphere originates from $NH_3$ oxidation (Novak and Fiorelli 2010) whereas the remainder of the anthropogenic $N_2O$ loading is considered to be from microbial denitrification.

Norway was one of the nations that ratified the Kyoto Protocol, devoting itself to produce a national inventory for $N_2O$ emissions from agricultural land use. Based on Tier 1 methodology (IPCC , 2007a), which relates estimated $N_2O$ emissions linearly to fertilizer sales, $N_2O$ accounted for 7.5% of Norway's aggregate GHG emissions in 2007 (statistics Norway, 2011), and was predominately caused by agriculture and manufacturing commercial fertilizer. The annual sales of N fertilizer in Norway have increased steadily until 1980 and remained above 10,000 tons since then (Fig. 1). Only a slight decline in the sales of N Fertilizer was recorded in 1998 when farm holdings that receive production grants were obliged to implement fertilization plans. Estimated $N_2O$ emissions in Norway in the period 1990 to 2010 are shown in Figure 2 together with other GHGs, the sum of which was slightly higher than the assigned amount under Kyoto protocol in 2010.

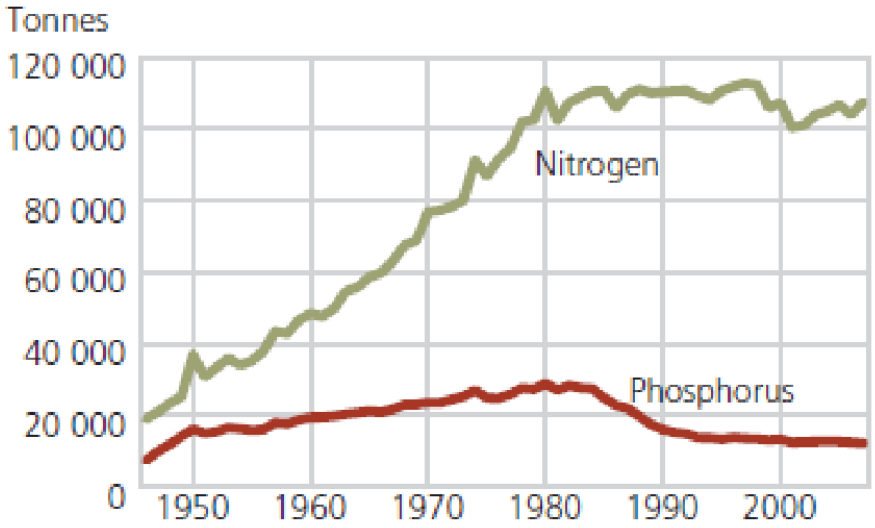


**Figure 1: Sales of nitrogen and phosphorus fertilizers in Norway 1946-2007** (source: Statistics Norway, ssb.no, 2011)

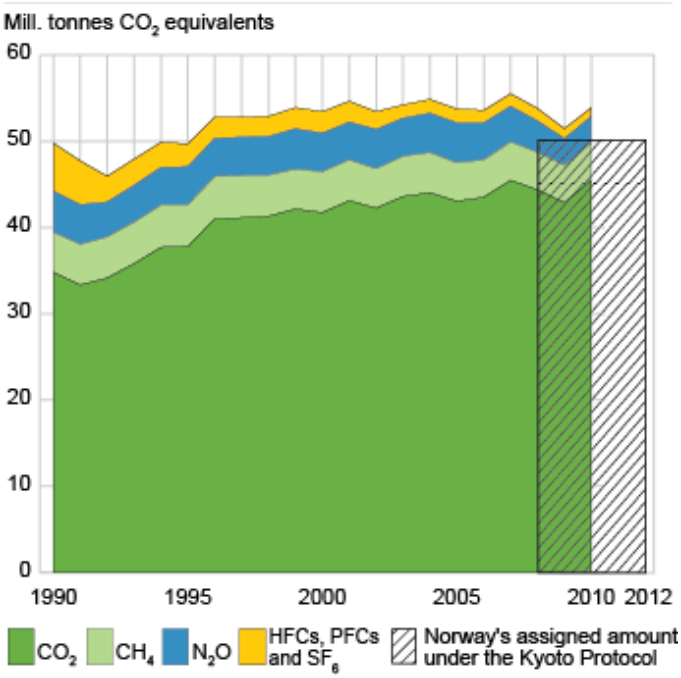


**Figure 2: Estimates of greenhouse gas emissions in the period of 1990-2010 and Norway's assigned amount 2008-2012 by the Kyoto protocol (shaded).** (source: Statistics Norway, ssb.no, 2011)

An emission factor as defined by IPCC is a "… rate of emission per unit of activity, output or input" (IPCC 2007b). For $N_2O$ emissions from soils, EFs based on N input have been reported to vary greatly both spatially and temporally. This is caused by different parameters that influence the microbial processes that lead to $N_2O$ production (Flechard et al., 2007). The default EF recommended by the IPCC is 1.25% of N applied as artificial and organic fertilizers and 2% of the N from the excreta of grazing animals (IPCC, 2001) which was later changed to 1% (IPCC 2006).

### 1.1.1 Microbial processes involved in the production and reduction of nitrous oxide ($N_2O$)

The production of $N_2O$ mainly results from two processes mediated by bacterial matabolism: 1) aerobic nitrification by autotrophic ammonia-oxidizing bacteria (AOB; genus *Nitrosomonas*) (Kowalchuk and Stephen 2001) or archea (AOA, *Crenarcheota*) (Leininger, Urich et al. 2006) where ammonium ($NH_4^+$) is oxidized into nitrite ($NO_2^-$) and from the oxidation of nitrite ($NO_2^-$) to $NO_3^-$ by nitrite oxidizers (genera *Nitrobacter* and *Nitrospira*)(Fig 3A); and 2) anaerobic denitrification by heterotrophic bacteria, where $NO_3^-$ acts as an electron acceptor and is reduced successively to $NO_2^-$, nitric oxide (NO), $N_2O$ and $N_2$ in the absence of oxygen (Skiba and Smith 2000), (Dalal, et al. 2003) (Fig. 3B). Wrage, et al. (2001) described a third pathway for the production of $N_2O$, so called "nitrifier denitrification" by which autotrophic nitrifiers reduce toxic $NO_2^-$ and NO to $N_2O$ and $N_2$ under suboxic conditions (Fig. 3A).

The $NO_3^-$ ion formed by nitrification can be easily lost by leaching or be subject to denitrification in the root zone (Giles 2005; Philippot, et al. 2009). Complete denitrification in soil closes the N cycle as soil nitrogen is returned to the atmosphere as

$N_2$. This process results in the depletion of nutrient N from soil but also mediates the removal of $NO_3^-$ from waters and sediments (Philippot et al., 2009).

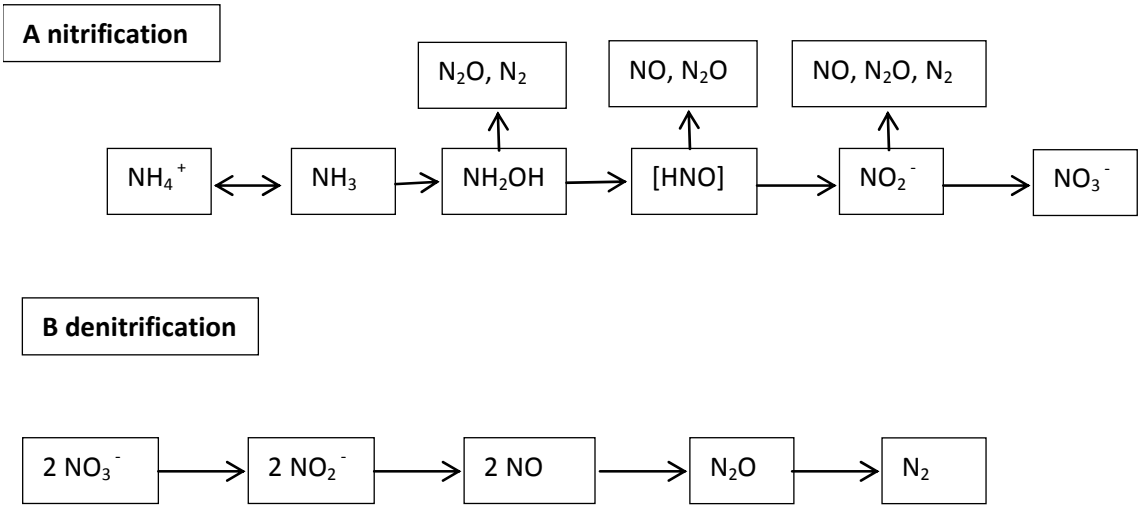


**Figure 3 Formation of gaseous N species (NO, $N_2O$, $N_2$) in the course of nitrification (A) and denitrification (B).** Adapted from Simek and Cooper (2006). $NH_4^+$, ammonium; $NH_3$, ammonia; $NH_2OH$, hydroxylamine; HNO nitroxyl; $NO_2^-$, nitrite; NO, nitric oxide, $N_2O$, nitrous oxide

### 1.1.2 Factors controlling $N_2O$ emissions.

$N_2O$ emissions in cultivated soils are controlled by a multitude of complex factors and their interactions. Both nitrification and denitrification are regulated by temperature, pH, availability and quality of C sources, and by soil moisture which influences the oxygen availability (Oenema, Velthof et al. 1997). The biogenic formation of $N_2O$ is also dependent on the form of the available mineral N which contributes to determining which process would be more dominant (Skiba and Smith 2000). Both nitrification and denitrification are influenced by soil temperature; water-filled pore space (WFPS), the form of available mineral nitrogen ($NH_4^+$ or $NO_3^-$), soil pH, and the alternation of dry and wet seasons. A consistent relationship with any of these factors alone could not be established with the rate of $N_2O$ emission flux, but the interplay of these factors was found to have a positive relationship with $N_2O$ emissions (Abdalla, Jones et al. 2010).

Denitrification requires availability of $NO_3^-$, whether it is applied directly as a fertilizer or produced by nitrification from $NH_4^+$ (Dalal, et al. 2003). However the availability of N alone does not account for $N_2O$ emissions (Rochette, et al. 2004). The water regime within the soil plays a crucial role for the release of reactive N as $N_2O$. When the water filled pore space (WFPS) reaches 60-70%, anaerobic conditions are created in zones of low $O_2$ diffusivity such as in soil aggregate cores, favoring denitrification. Above 80-90% WFPS, transport of $N_2O$ out of the soil becomes limiting and $NO_3^-$ is more completely reduced to $N_2$. Soil pH below 3.5 reduces denitrification (Dalal et al., 2003). Land use, crop type, soil temperature and oxygen pressure also influence the magnitude of $N_2O$ emissions (Granli and Bockman 1994). Climate and soil conditions have more influence on $N_2O$ emission than N fertilization (Jungkunst, et al. 2006).

Given the complex regulation of $N_2O$ emissions, emission factors for $N_2O$ are expected to vary greatly with fertilizer regime (fertilizer type, quantity and timing), type of crops and management practices (tillage, grazing), all of which result in spatial variability on a regional level. Seasonal and annual weather fluctuations on the other hand result in temporal variability (Kuikman et al., 2006). The uncertainty generated with respect to emission factors of $N_2O$ is thus attributed to the interplay of agronomic regime and variation in climatic conditions from one season to another, and from one year to another (Skiba and Smith, 2000). Given this variability and the resulting uncertainty about $N_2O$ emissions on regional or national scales, the simple Tier 1 emission factor based on N application rate should be disintegrated and replaced by site specific models (Tier 2 and 3, IPCC 2007) that include the different factors that influence $N_2O$ emission (Skiba and Smith, Jungkunst et al., 2006).

### 1.1.3 Nitrous oxide consumption

The main sink for $N_2O$ is stratospheric destruction by reaction with excited oxygen atoms (Crutzen 1981, Dalal et al., 2003). Unlike carbon, which can be sequestered by soils, little is known about possible $N_2O$ sinks in soil-plant systems. Ecosystems influence the lifetime of $N_2O$ either as sources or as sinks depending on a variety of climatic conditions and soil properties (Field, Lobell et al. 2007). High WFPS values

(>80 %) can result in reduction of $N_2O$ to $N_2$ by the denitrification enzyme $N_2O$ reductase ($N_2OR$) (Dalal et al. 2003; Chapuis-Lardy et al. 2007). Similar to $N_2O$ production, $N_2O$ consumption by biological reduction in soils depends on a wide range of factors interacting with each other. Mosier et al. (1998) suggested that soil uptake of atmospheric $N_2O$ is not significant enough to be included in the $N_2O$ budget of a given agricultural system. However, Chapuis Lardy et al. (2007) countered this argument by suggesting that negative $N_2O$ flux, i.e. net $N_2O$ uptake from the atmosphere should not be neglected.

### 1.2 Grasslands and $N_2O$ emission

Grasslands in Norway (both temporary and permanent) represent 64% of the overall agricultural area and 2.15% of the overall land area (Tab. 1). Grazing pastures have been recognized as important sources for $N_2O$ emissions (de Klein, et al. 2003)

**Table 1 : Pasture area in relation to agricultural land, and the overall land area in Norway** (source: FAOSTAT 2009)

| item | 2009 | |
|---|---|---|
| **Country area** | 32378.00 | 1000 Ha |
| **Land area** | 30547.00 | 1000 Ha |
| **Agricultural area** | 1014.38 | 1000 Ha |
| **Temporary meadows and pastures** | 482.90 | 1000 Ha |
| **Permanent meadows and pastures** | 174.80 | 1000 Ha |

In order to devise mitigation strategies for $N_2O$ emission in agricultural ecosystem, it is important to consider the management regime and its specific C and N cycling on a farm level (Ledgard, et al. 2009). Grasslands, provide a number of environmental services such as supporting biodiversity, animal welfare, and reducing $NO_3$-N leaching,

while increasing protein self-sufficiency within the farm system (Peyraud et al., 2010). Grasslands have been also reported to have the potential to sequester carbon even more than trees can (Pearson and Ison, 1997). In addition, well managed grassland systems can provide a number of economic benefits such as competitiveness, high product quality and efficient energy use, as well as social benefits such as overall wellbeing of farm workers. Grassland systems define the landscape in which they exist (Hopkins and Wilkins 2006).

Managed Grasslands cover 3 billion hectares and have been implicated as "a key contributor to $N_2O$ emissions" (Lee et al. 1997; (Klumpp, Bloor et al. 2011). According to the IPCC Guidelines (1996), grazed pastures emit 1600 Gg $N_2O$-N per year, which amounts to 28% of the global anthropogenic $N_2O$ load (Denmead, Leuning et al. 2000). GHG fluxes in grassland-based agricultural systems are dependent on pasture management practices (Soussana,et al. 2004). Grasslands with no N fixing legumes are dependent on the input of external N fertilizer to improve productivity. In this case the applied N may exceed the plant needs, resulting in N losses through $NO_3$ leaching or volatilization of $NH_3$, NO, and $N_2O$ (Flechard, et al. 2007; Soussana, et al. 2007).

Grazing systems on legume/grass pastures can be a good example for efficient N cycling, where N is fixed by legumes, taken up by grass, consumed by grazing animals, and returned to the soil through animal excreta, and through crop residues of legumes (Ledgard, Sprosen et al. 2001). Biological nitrogen fixation (BNF) in grass-clover swards can be equivalent to 150-250 kg N $ha^{-1}$ $a^{-1}$, thus reducing the need for applying external fertilizer, and saving the energy required to manufacture artificial N fertilizers (Peyraud and Delaby, 2006). However, the presence of legumes in grasslands has been reported to contribute to the increase in anthropogenic $N_2O$ emissions through enriching the soil with fixed N, or through the mineralization of N-rich residues or root exudates (Denmead et al., 2000). High $N_2O$ losses that have been observed from soils under legume pastures were attributed to biological nitrogen fixation (Bouwman 1996). Niklaus et al. (2006) argued that $N_2O$ emissions increase in the presence of legumes. This could be mediated by two mechanisms: 1) supplying the soil microbial community with N-rich crop residues and exudates, and 2) supporting rhizobia denitrification (Ohara and

Daniel 1985). Some of the symbiotic bacterial strains capable to fix $N_2$ (genus *Rhizobia*) have been reported to be denitrifies reducing excess $NO_3^-$ to $N_2O$ (Bedmar, et al. 2005). However the contribution of rhizobia to total denitrification compared to other soil microbes was considered negligible in other studies (Garcia-Plazaola, Becerril et al. 1993). The main source of $N_2O$ emissions associated with legumes appears to be the N released from root exudates and the decomposition of crop residues after harvest, rather than N fixation itself (Rochette and Janzen 2005). A recent study concluded that N fertilization has a bigger impact on $N_2O$ emissions compared to N fixing (Zhong, Lemke et al. 2009)

Legume/grass pastures use N more efficiently than fertilized grass (Boller and Nosberger 1987). More recent studies showed that clover-grass mixtures resulted in a smaller $N_2O$ flux than N fertilized grass, which has been the basis for the argument that legume-grass pastures can be used as a mitigation strategy for $N_2O$ emissions (Carter and Ambus 2006).

In grazing pastures animal excreta are a major contributor to increased $N_2O$ flux from soil. Urine patches result in hot spots for $N_2O$ release immediately after urine is introduced into the soil (Flessa, Dorsch et al. 1996). Urine patches from grazing animals are highly localized N applications (Van Groeningen et al., 2005) that cause an immediate but transient increase in $N_2O$ flux. However, the default $N_2O$ emission factor set by IPCC (1997) for animal urine in pastures (2%) was considered too high and was corrected to 1% (Bouwman, et al. 2002; Van Groeningen et al. 2005). According to Oenema et al. (1997), 70 % of N in animal urine is in the form of urea. Urea is quickly hydrolyzed to $NH_4^+$ in soils, which is then subject to nitrification, and eventually denitrification (Bolan,et al. 2004; Luo, et al. 2008). Urine deposition results in immediate high $N_2O$ flux where nitrification is the most important process triggered by the enhanced availability of $NH_4^+$. During nitrification, the production of $NO_3^-$ inhibits the activity of bacteria that convert $NO_2^-$ to $NO_3^-$, resulting in a temporary accumulation of NO that is further oxidized into $N_2O$ (Oenema et al., 1997). The effect of animal urine on increased $N_2O$ flux from soil is enhanced by soil compaction from animal treading and

$O_2$ depletion by easily degradable carbon from animal dung (van Groeningen et al., 2005).

### 1.3 Multisward project and the main hypothesis

The present study was conducted in the framework of the Multisward Project, an EU funded fp7 project under the theme of knowledge based bioeconomy, KBBE. The overall aim of the project is to " … assess the performance of multi-species swards (MSS) in terms of plant productivity and animal nutrition over a range of environments and determine the most appropriate mixtures according to the soil and climatic conditions" (Marchoux 2010).

The project embarks from recent findings supporting the assumption that species richness results in higher and more even yields (Kirwan,et al. 2007; Nyfeler, et al. 2009). Hence, legume-grass pastures are expected to result in more evenly distributed seasonal production curves on the longer term given the increased yields that result from legume grass interactions. According to the Multisward project description, "… the true functional benefit of increasing plant diversity may only be appreciated when multiple ecosystem processes are considered simultaneously". Thus, the present study focuses on the role of clover density within multispecies swards for $N_2O$ emissions from urine patches. This includes studying the effect of clover on soil N and N yield in companion grass.

Previous work by Klumpp,et al. (2011) showed no difference in the effect of clover density on $N_2O$ emissions. In the present study, artificial urine was applied to simulate the condition of grazed pasture, using different proportions of clover ranging between 0% and 100%. The experiments were conducted at UMB and involve species combinations of *Phleum pratense, Lolium perenne or Festuca arundinacea, Trifolium repens and Trifolium pretense* (Tab. 2). The expected functional interaction studied in my experiment is the effect of clover species and density on plant soil N uptake (since

$N_2$ fixation will be ceased as a result of N deposition in urine) in competition with soil microbial N turnover leading to $N_2O$ emissions.

# 2. Materials and Methods

## 2.1 Setting up the experiment

### 2.1.1 Site description

The $N_2O$ measurements reported here were conducted in a fully factorial plot experiment established on the experimental farm “Østrevoll” of the Norwegian University of Life Sciences in 2010. The experimental field is located at 59° 39’ 54 21 "N and 10° 44' 21’’E, 88 meters above sea level (Follokart.no). The field is lying on a slope with 3 meters hight difference. The soil is as naturally poorly drained silty clay loam (Bakken et al, 2006).

Gras-clover mixtures were sown on June 23$^{rd}$, 2010 using barley as a cover crop, as it is usual in Norway. Two grass species and two clover species were sown in different mixtures, ranging from 0% to 100% clover. Each of the four species was sown in pure stands Lp_m, Fa_m, Tr_m and Tp_m (where “m” refers to monoculture, Tab. 3), and in mixtures Lp_d, Fa_d, Tr_d and Tp_d as the dominant species (67%) whereas the remaining three species were sown at 11%, (where “d” refers to dominant, Tab. 3). In the centroid treatment, each species was sown at 25%. Figure 3 shows the northern part of the field in August. There were two cutting regimes: 3 harvests per year as common in this region and simulated grazing (5 harvests/year). Plots were sown at two seeding densities 10 kg ha$^{-1}$ (plots 1-9) and (20 kg ha$^{-1}$ plots 10-18).

**Figure 4 Plots in the northern part of the experimental field in August 2011. (*photo by Marina Blekken*)**

**Table 2: Names of sown species and the cultivars used for each.**

| **Scientific name** | **English name** | **Cultivar** |
|---|---|---|
| *Lollium perenne* | Ryegrass | Fagerlin |
| *Festuca arundinacea* | Tall fescue | Kora |
| *Trifolium repens* | White clover | Milkanova |
| *Trifolium pratense* | Red clover | Lea |

**Table 3: Distribution of sown species per treatment (% clover)**

| Working name | Lp | Fa | Tr | Tp | Treatment |
|---|---|---|---|---|---|
| Lp_m | 100 | 0 | 0 | 0 | pure ryegrass |
| Fa_m | 0 | 100 | 0 | 0 | pure fescue |
| Tr_m | 0 | 0 | 100 | 0 | pure white clover |
| Tp_m | 0 | 0 | 0 | 100 | pure red clover |
| CC | 25 | 25 | 25 | 25 | centroid |
| Lp_d | 67 | 11 | 11 | 11 | dominant rye grass |
| Fa_d | 11 | 67 | 11 | 11 | dominant fescue |
| Tr_d | 11 | 11 | 67 | 11 | dominant white clover |
| Tp_d | 11 | 11 | 11 | 67 | dominant red clover |

$N_2O$ flux measurements were set up on selected plots for simulated grazing only (Tab. 4, Fig. 5). The experiment preparations started on the 12$^{th}$ of August 2011, after the fourth harvest (3$^{rd}$ of August.) The experiment was started by visual assessment of the sward composition. Then, areas of about 70*140 cm were demarcated in 2-3 plots per treatment. A part of each of the selected plots was used for setting one frame for the static flux chambers (see below) and the rest was used for soil sampling (Fig. 5). A total of 30 frames were distributed, three frames per treatment, apart from the centroid which received 6 frames.

**Table 4: Different treatments and the distribution of frames on field plots. N indicates plots on the northern part of the field, while SW indicates the southwestern part of the field.**

| Working name | Plots | frames |
|---|---|---|
| Lp_m | N1-SW10 | 5, 19,26 |
| Fa_m | N2, N11 | 8 , 13, 22 |
| Tr_m | N3-N12 | 10,23,28 |
| Tp_m | N13 | 9, 12, 16 |
| CC | N14, N5 | 6,14,15/18,24,25 |
| Lp_d | N6-N15 | 7,11,17 |
| Fa_d | N7- SW16 | 2,20,27 |
| Tr_d | N8-SW8- SW17 | 3,4,30 |
| Tp_d | SW9-N9 | 1,21,29 |

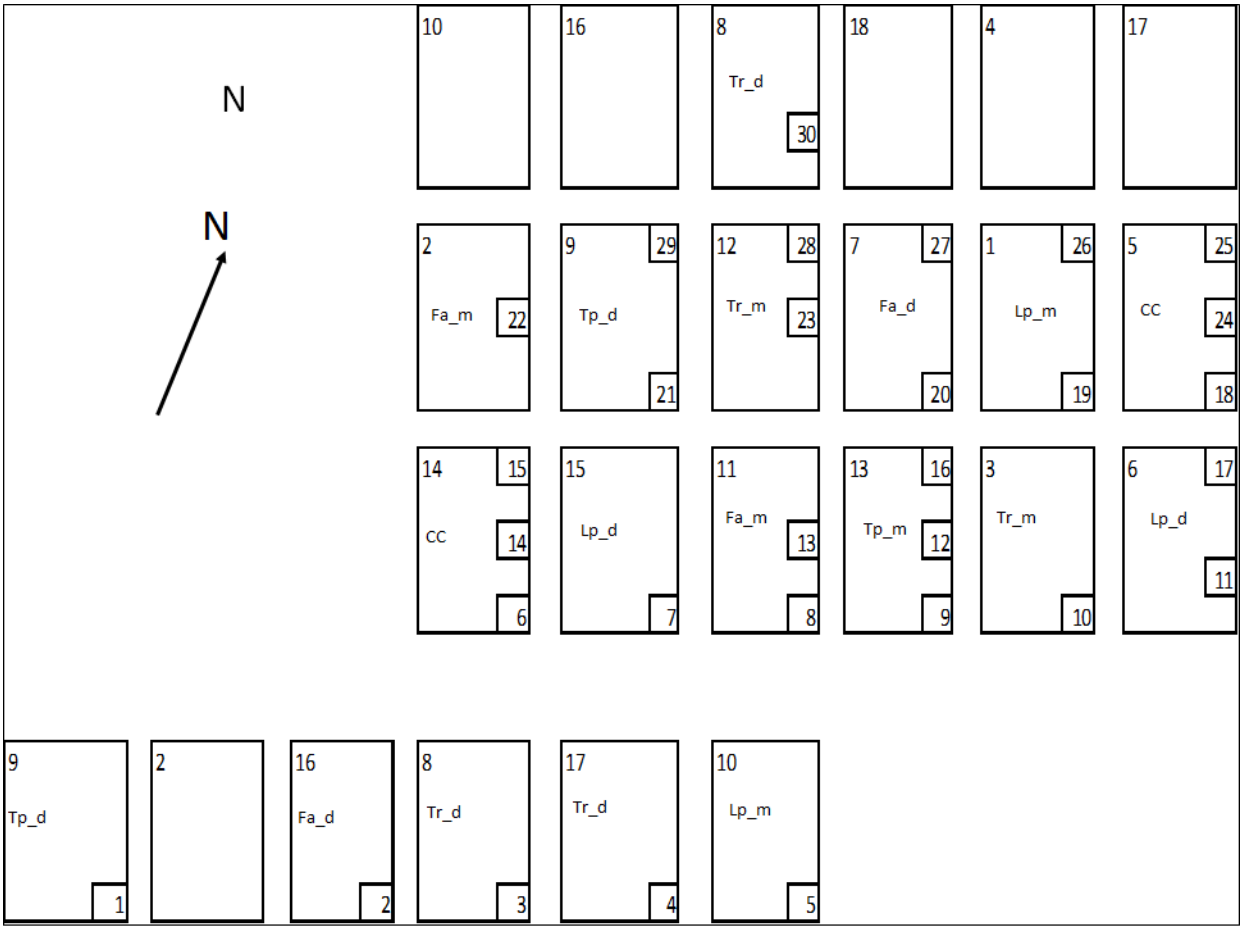


**Figure 5: Distribution and enumeration of plots and frames of the Multisward $N_2O$ trial according to treatment (Table 3). The arrow indicates the North direction**

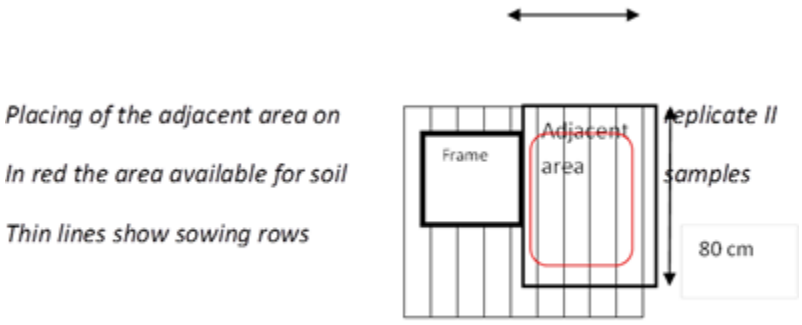


**Figure 6: Areas designated for soil sampling (65 cm x 80 cm) were adjacent to the frames**

### 2.1.2 Artificial urine

On August 22nd, 2011, artificial urine was applied at a rate of 5 L $m^{-2}$, adding 50 g N $m^{-2}$. The components of artificial urine are given in Table 5 modified from Ambus, et al. (2007).

**Table 5: Chemical composition of artificial urine**

| Artificial urine | Amount | Nitrogen g $L^{-1}$ |
|---|---|---|
| Urea | 21.74 g $L^{-1}$ | 10.0004 |
| $KHCO_3$ | 12.8 g $L^{-1}$ | |
| KCl | 10 g $L^{-1}$ | |

The solution was added to the 65 x 80 cm area adjacent to the frame and the area of the metal frames (0.250 $m^2$). Each frame received 1.3 L of artificial urine solution. 2.6 L of the same solution was added to the area adjacent to the frame. For practical reasons we prepared a concentrated solution and used 100 ml concentrate solution per frame and 200 ml per adjacent area which was diluted to 1.3 and 2.6 L, respectively, directly before the application.

## 2.2 Sampling

### 2.2.1 Flux Sampling

Prior to sampling $N_2O$ emission flux, permanent aluminium frames were driven 5 cm into the soil. The frames were equipped with a group on top, which was filled with water to ensure airtight connection between soil and flux chamber during flux measurement. For each flux measurement, vented, closed static chambers (51.2 x 52.2 x 20 cm) were

deployed onto the preinstalled frames from which four gas samples were drawn at 0, 15, 30 and 45 min deployment time using a 20 ml disposable syringe which was connected to the chamber volume with a tube. Prior to taking a gas sample, the plunger of the syringe was pumped 5 times to mix the gas inside the chamber, before injecting a 20 ml gas sample into a 12 ml evacuated vial. Flux measurements were conducted twice weekly, and once a week after the 5$^{th}$ harvest. Soil and chamber temperatures were recorded at the start and end of the deployment time.

#### 2.2.2 Plant samples

The botanical composition of the swards in the different treatments was determined after the 5$^{th}$ harvest on the 13$^{th}$ of September. For this, plants inside the frame were cut 5 cm above soil and sorted into following groups: *L. perenne, F. arundinacea, T. repens, T. pratense*, others. The dry matter mass of each group was registered after drying at 60 $^{0}$C. Thereafter, a subsample of each group was chopped and ground for analysis of total N content in each of the three replicates collected per treatment.

#### 2.2.3 Soil samples

Two soil cores (30 mm diameter, 20 cm depth) were taken from each area adjacent to the frames and pooled to composite sample for each treatment. For the centroid, two separate composite samples were taken. The first sampling was conducted before urine application, thereafter about once a week, for a total of 9 sampling dates (Tab. 4). The samples were transferred to the laboratory, homogenized manually and 40 g of each sample were placed into a 150 ml Duran glass and frozen (-18$^{o}$C) for later extraction. Another 10 grams of fresh weight soil were suspended in 25 ml distilled water and

shaken horizontally for 30-60 minutes, before measuring the pH by with an ORION SA720 electrode pH meter connected to a Orion ROSS Ultra pH Electrode. Water content was measured gravimetrically by taking 20 g of soil in glass beakers, determining weights, and drying the sample at 105$^{o}$C for two days.

For extraction of $NO_3^-$ and $NH_4^+$, the frozen samples were thawed and 50 ml 2 M KCL solution was added immediately. After shaking the suspension for 1 hour, it was poured into funnels lined with Whatman filter paper, (Blauband 589/3 ø 125 mm) and the solution was allowed to drip in plastic tubes. The tubes were sealed and labelled together with two blanks (KCl solution without the soil sample) and were kept in the freezer before sending to analysis.

Due to an error with the attribution of numbers to the frames in the field, most samples were excluded from the first five samplings (before the 30$^{th}$ of September), leaving only four samples from each sampling date which could be assigned unequivocally to each of the treatments given in table 3. Samples from the first five sampling dates had to be reconstructed according to the contributions of different treatments in the mixed sample. They were assigned to one of the following three classes “Grass”, “White clover-dominated” and “Red clover-dominated” (Tab. 6). In addition, one “pure” treatment could be reconstructed, which was the treatment dominate white clover (Tr_d; Tab. 3) and is the only treatment that had intact soil sampling throughout the whole trial period. Samples which could not be assigned to any of the four “classes” were discarded.

**Table 6 Composite soil samples obtained in the period before 30th of September. the Grass sample represents ryegrass and tall fescue treatments. WC and RC represent white clover and red clover-dominant treatments, respectively. Numbers following the treatment names denote frame numbers (Fig 4) from which samples were retrieved.**

| **Reconstructed classes** | **treatments** |
|---|---|
| Grass | Lp_m 5 + Fa_d 27 + Fa_m 22 |
| WC | Tr_m 23 + Tr_m 28 + Tp_d 29 |

| | |
|---|---|
| RC | Tp_m 12 + Tp_m 16 + CC 6 |

## 2.3 Analysis of Samples

### 2.3.1 Plant and Soil samples

The determination of total N was done according to the Dumas method, described in Bremmer and Mulvaney (1982). Sample material was ground by a mortar before weighing. About 200 mg of each sample was weighed in a tin foil (100-150 mg for samples high in organic material). Samples were analyzed by a combustion CN analyser (Leco CHN 1000) at the Institute for Plant and Environmenmtal Sciences, UMB. During the analysis (at 1050°C), nitrogen oxides are reduced with the help of copper to $N_2$. The concentration of $N_2$ gas is determined by a thermal conductivity detector (TCD).

### 2.3.2 Gas Analysis

The mixing ratios of $N_2O$ and $CO_2$ in chamber air over deployment time were determined by gas chromatography (GC) in the GHG-laboratory of the UMB-Institute for Plant and Environmental Sciences. The GC used was a Agilent 7890A (USA), equipped with a 250 µl sampling loop mounted on a pneumatic 6-port valve, a packed Haysep-precolumn for back flushing and a 30 mm long 0.53 mm diameter Poraplot U capillary column separating $CO_2$, $CH_4$ and $N_2O$ from air. Helium was used as a carrier gas. $CO_2$ was measured by a thermal conductivity detector (TCD) and $N_2O$ by an electron capture detector (ECD). The latter operates at $340^o$ C with $Ar/CH_4$ (90/10 vol%) as make up gas. Data acquisition and peak integration was done by EZchrome software, and the auto sampler was operated by in-house software (Molstad, et al.

2007). A 120 ml bottle filled with a calibrated standard close to ambient air was included into the measurement sequence after each 8 analyses of unknown samples and used for calibration and drift correction. The vials were organized systematically according to the frame number in groups of four in order to see the increase in gas emission over time.

Emission rates of $N_2O$ and $CO_2$ were calculated from the increase of concentrations over time. For this, gas concentrations were plotted in Excel and inspected individually for each measured flux. The slope (ppm $min^{-1}$) was calculated by least square regression and converted to flux rates using the following equation:

$$F_{N2O} = (dN_2O/dt \times V/M_v) \times 60 \times f/A$$

Where $F_{N2O}$ is the $N_2O$ flux (µg $N_2O$-N $m^{-2}h^{-1}$), $dN_2O/dt$ the change of $N_2O$ concentration in the chamber (ppbv $min^{-1}$), V the total volume of the chamber (L), A the area covered by the chamber ($m^2$), $M_v$ the molecular volume at chamber temperature (moles $L^{-1}$) and F a conversion factor (0.0028).

#### 2.3.3 Ammonium and nitrate in soil

$NO_3^-$ and $NH_4^+$ concentrations were analysed by flow injection analyses at UMB-IPM. $NO_3^-$ is reduced by a cadmium amalgam to $NO_2^-$. The reduction takes in a column (Jones reducer) in the presence of ammonium as a buffer solution and forms a complex with cadmium ions. Nitrate reacts with a strongly acidic solution (pH between 1.5 and 2) with sulfanilamide forming a double bond, which produces the azo-compound N-(l-nafthyl)-ethylendiamin. The absorbance of the latter compound is measured at wavelength of 545 nm. The procedure was carried out according to the protocol described in the Norwegian standard for determining the sum of nitrate and nitrite nitrogen (NS 4745 1975).

For ammonium analysis, $NH_4^+$ reacts with a strong alkaline solution (pH 10,8 to 11,4) with chlorine to produce monochloramine which in presence of salicylic acid and excess hypochloride produces indo-phenole blue. Absorbance of the latter is measured at

wavelength 630 nm. The reaction is catalyzed by penta-cyanonitrosulphate (nitroprussid). The analysis was performed according to the protocol described in the Norwegian standard for determining Ammonium nitrogen (NS 4746 1975). The analysis work was performed at the soil laboratory at IPM.

#### 2.3.4 Statistical analysis

All data were compiled and linear regression was performed in Excel. One way ANOVA and Fischer test for significance were done using Minitab.

# 3. Results

### 3.1 $N_2O$ emission response to urea application

Figure 7A shows $N_2O$ fluxes in the experimental plots measured August through October 2011. $N_2O$ emissions were moderate in magnitude in the beginning of August after the fourth cut (< 100 µg N $m^{-2}$ $h^{-1}$) and increased markedly after the application of artificial urine on August 22nd. However, increase in $N_2O$ emission rates was moderate directly after fertilization presumably because it took some time for the urea to be hydrolyzed and nitrified before becoming subject to denitrification. The urea was applied in a solution equivalent to ~50 mm precipitation, but as seen from Fig. 7B and C, $NO_3^-$ concentrations were low at the time of urea application (< 1 g N $m^{-2}$ 0.2 $m^{-1}$), apparently limiting denitrification in the wet soil after fertilization. $N_2O$ emissions increased more strongly Aug. 29th after extensive rainfalls resulting in WFPS > 50% (Fig. 7C) along with measurable increase in $NH_4^+$ and $NO_3^-$ concentrations in the soil (Fig. 7B, D). This resulted in a first $N_2O$ emission peak on Aug. 26th for all treatments (300-700 µg N $m^{-2}$ $h^{-1}$) except Tp_m, which peaked later on Aug. 30th. Emission peaks during this period were highest in Tr_m, Tr_d, Tp_m and Tp_d, in other words plots dominated by clover tended to show stronger $N_2O$ emission response to urea application than grass dominated plots, although this difference was not significant.

A second emission peak was observed on the Sept. 8th for Tp_m (1008.3 ± 41.4 µg N $m^{-2}$ $h^{-1}$), Tp_d (1137 ± 766 µg N $m^{-2}$ $h^{-1}$) and Fa_d (946.8 ± 585 µg N $m^{-2}$ $h^{-1}$), where-as $N_2O$ emission for the other treatments remained at rates below 500 µg N $m^{-2}$ $h^{-1}$. Also this emission peak seemed to be triggered by extensive rainfalls, but due to lack of soil samples for this period, no WFPS values are available. Towards the end of September, emissions started to decline gradually. This decline was concomitant with declining concentrations of $NH_4^+$ and WFPS until the end of the sampling period, when flux rates leveled off to background rates similar to those observed before the application of urea.

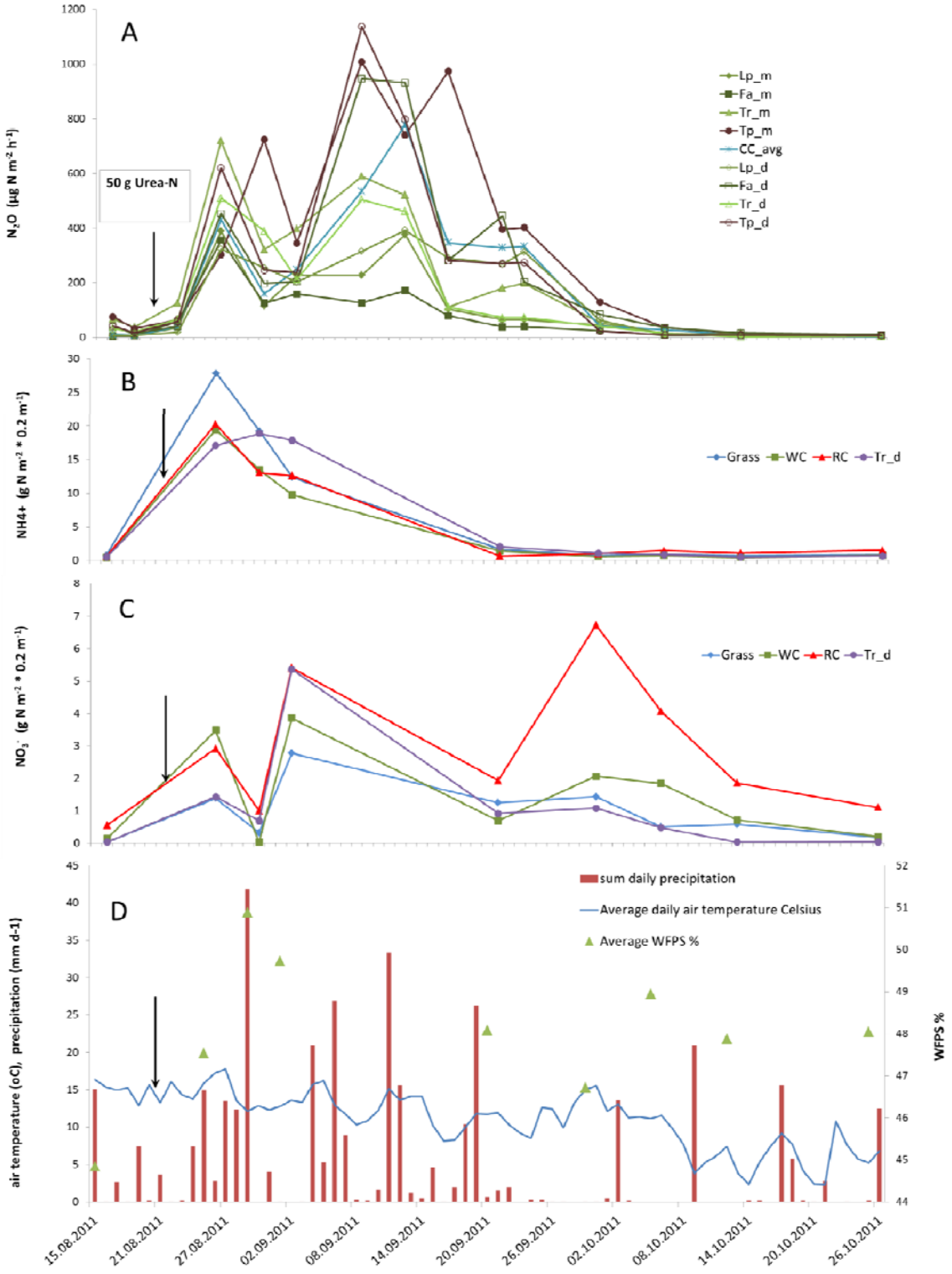


**Figure 7: Time course of A) $N_2O$ emission (µg $m^{-2}$ $h^{-1}$), B) soil $NH_4^+$ for reconstructed samples (g N $m^{-2}$ 0.2 m depth), C) soil $NO_3^-$ for reconstructed samples (g N $m^{-2}$ 0.2 m depth) and D) rainfall (mm $day^{-1}$), air temperature (°C) and water filled pore space (%). Treatment Tr_d denotes the original treatment with dominant *T. repens*.**

### 3.2 Soil N dynamics

Due to a mistake in pooling soil samples from the various plots, 3 new "treatment groups" were constructed, representing plots dominated by grass (mainly tall fescue), white clover and red clover (Tab. 6). Only Tr_d could be assigned unequivocally to soil pooled from one of the original treatments. $NH_4^+$ concentrations in soil peaked right after the application of artificial urine (Fig. 7B) reaching the highest concentration under grass (27.9 g $m^{-2}$) and somewhat lower concentrations in treatments dominated by white (19.5 g $m^{-2}$) and red clover (20.3 g $m^{-2}$). The Treatment Tr_d peaked one week later (18.9 g $m^{-2}$) than the other treatments. $NH_4^+$ declined rapidly and reached baseline levels at 21$^{st}$ of September. The soil pH did not change after the addition of artificial urine (data not shown).

$NO_3^-$ concentrations started rising gradually after the application of artificial urine and remained well below $NH_4^+$-N values (Fig. 7C; note different scale of y-axis in figures 7B and C). A sharp decline in soil $NO_3^-$ was observed following heavy rainfall on Aug. 29$^{th}$ (41.8 mm) after which $NO_3^-$ started to rise again, reaching a second peak 3 days later. A third peak in $NO_3^-$ content was recorded on Sept. 30$^{th}$, after a longer dry period (cf. Fig. 7C and D), without leading to increased $N_2O$ emission rates (Fig. 7C).

Figure 8 compares $N_2O$ flux averaged for grass-, white clover-, and red clover-dominated plots as well as for treatment Tr_d with reconstructed mineral N dynamics (Tab. 4) from start of the experiment until Sept. 23$^{rd}$. Increase in $N_2O$ emission rates after urea application was weakest in grass dominated plots. Among clover-dominated plots, urea-induced $N_2O$ flux was clearly higher in white clover-dominated plots and the treatment Tr_d than in red clover-dominated plots. However, $N_2O$ emission flux in RC-dominated plots was highest towards the end of the trial period despite similar values of mineral N in all treatments.

Mineral N data for the 9 original treatments (Tab.3) were only available for the last four sampling dates and are shown in figure 9 except for the "centroid" treatment. The gradual decline in $N_2O$ emission corresponded to the decrease in $NO_3^-$ for most

treatments whereas $NH_4^+$ remained relatively low. Towards the end of the trial (30/09) soil $NO_3^-$ was higher under grass mixed with legumes treatments Lp_d and Fa_d (3.83 and 2.58 g $m^{-2}$, respectively) than for the pure grass stands Lp_m and Fa_ m (1.67 and 0.82 g $m^{-2}$). On the other hand $NO_3^-$ under pure clover dominant treatments was comparable with that under pure grass stands (1.34 and 0.31 g $m^{-2}$) and lower than pure clover treatments Tr_m and Tp_m (2.37 and 4.58 g $m^{-2}$).

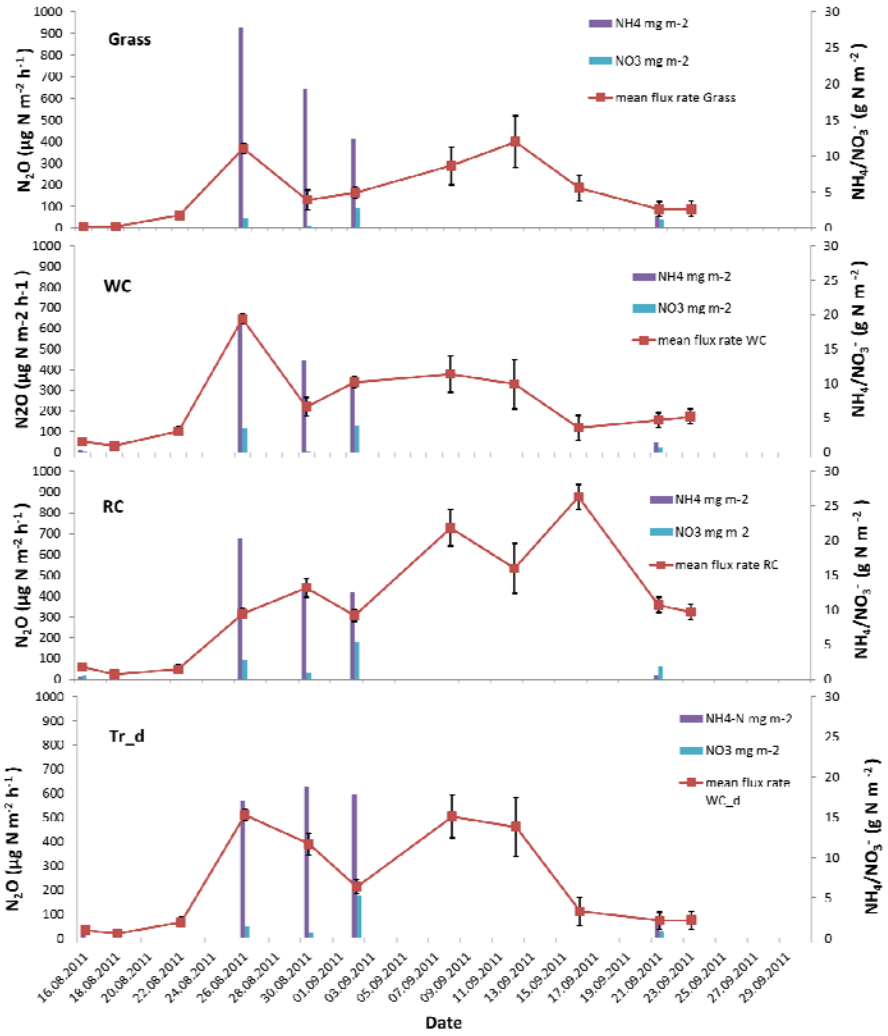


**Figure 8: Mean (n=3; error bars: SE) $N_2O$ emission rates (µg N $m^{-2}$ $h^{-2}$) and $NH_4^+$ and $NO_3^-$ (mg N $m^{-2}$ ) before 30 Sept for the three reconstructed treatments grass, white clover-dominated (WC), red clover-dominated (RC) and the original treatment white clover-dominated (Tr_d)**

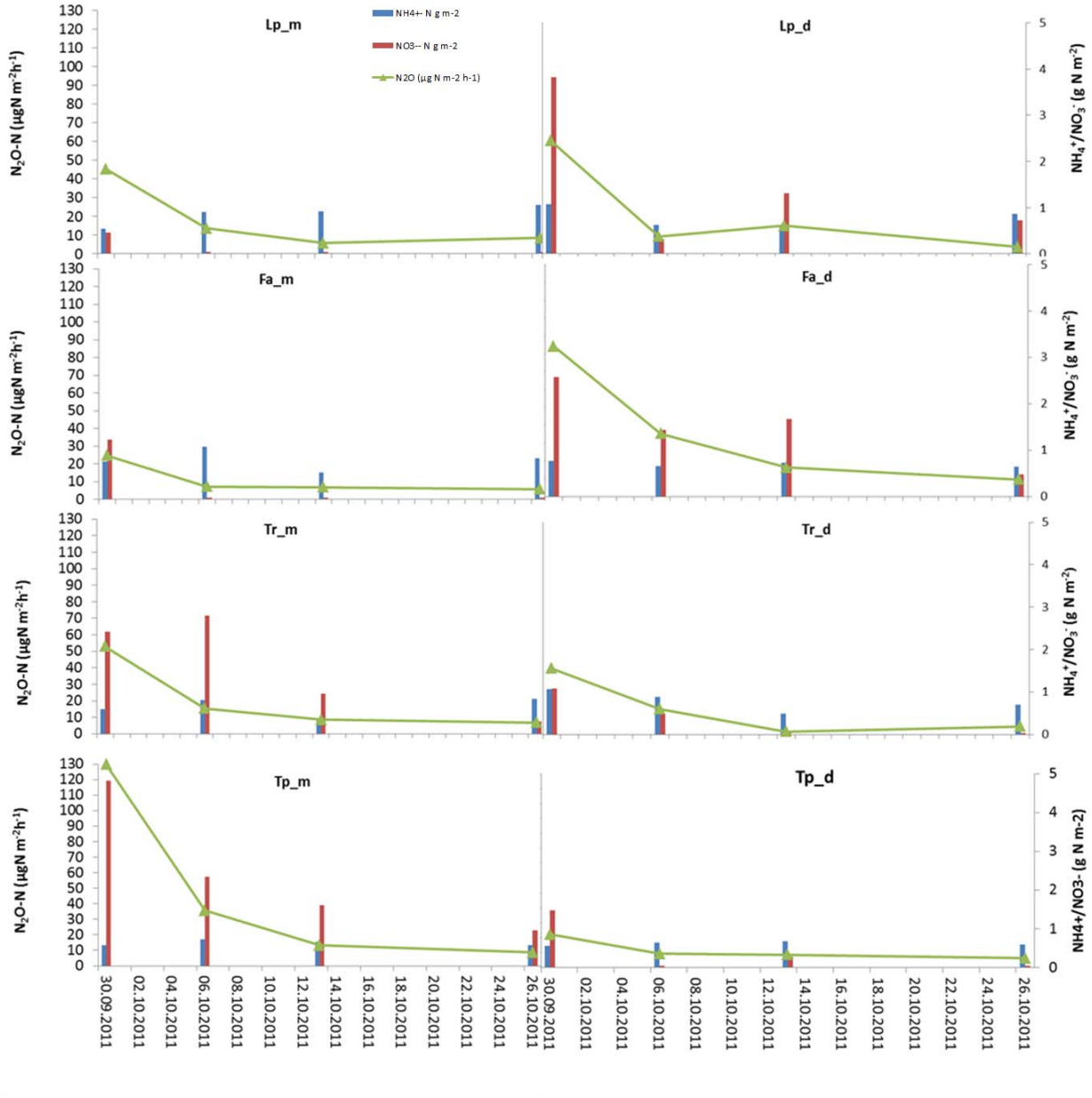


**Figure 9 Mean (n=3) $N_2O$ emission rates (µg N $m^{-2}$ $h^{-1}$) and $NH_4^+$ and $NO_3^-$ (mg N $m^{-2}$) after 30th of September for all treatments except the centroid treatments.**

### 3.3 Cumulative $N_2O$ emissions

The total amount of $N_2O$ released during the trial period (71 days) was calculated for all 9 treatments (Fig. 5) and is presented in figure 10. Tp_m (0.42 g $m^{-2}$) released significantly more $N_2O$ than Lp_m, Fa_m, Lp_d and Tr_d (0.16, 0.17, 0.34 and 0.26 g $m^{-2}$, respectively). The standard error was high for treatments Tp_d, Tp_m and Lp_d, preventing any conclusion about the effect of clover species and density on the basis of cumulative emission. Treatments with pure stands of ryegrass (Lp_m) and tall fescue (Fa_m) were associated with the lowest $N_2O$ emissions (0.17 ± 0.04 g N $m^{-2}$and 0.16 ± 0.08 g N $m^{-2}$, respectively). In grass dominated treatments (Lp_d and Fa_d), $N_2O$ emissions were higher (0.34 ± 0.15 g N $m^{-2}$ and 0.54 ± 0.04 g N $m^{-2}$, respectively) than in the pure grass treatments. Highest cumulative emissions were found in the tall fescue dominant treatment followed by red clover pure stand (Tp_m; 0.42 ± 0.34 g N $m^{-2}$) and the red clover dominated treatments (Tp_d; 0. 40 ± 0. 33 g N $m^{-2}$).

Cumulative flux rates for individual replicates within each treatment of the trial were compared (Fig. 11). For most treatments, it was observed that one curve would be higher than the other two. This pattern was observed for treatments Lp_m, Fa_m, Tr_m, Fa_d and Tp_d. When data for the different replicates were tested by one way ANOVA, significant differences were found between the frame associated with the higher emissions and the two frames with similar emissions for treatments Tp_d, Fa_d and Tr_m. The frames with the higher emission were usually the ones lying on flat terrain within the experimental field which was sloping to the northwest. Therefore, replicates were categorized according to topography (Fig. 12), where frames lying on a flat surface (frame numbers 1-10) seemed to produce higher emissions than the ones from the same treatments situated on a slope (frame numbers 17-30). The effect of slope was not evident for treatments CC1, CC2 and Tp_m (see Fig. 5).

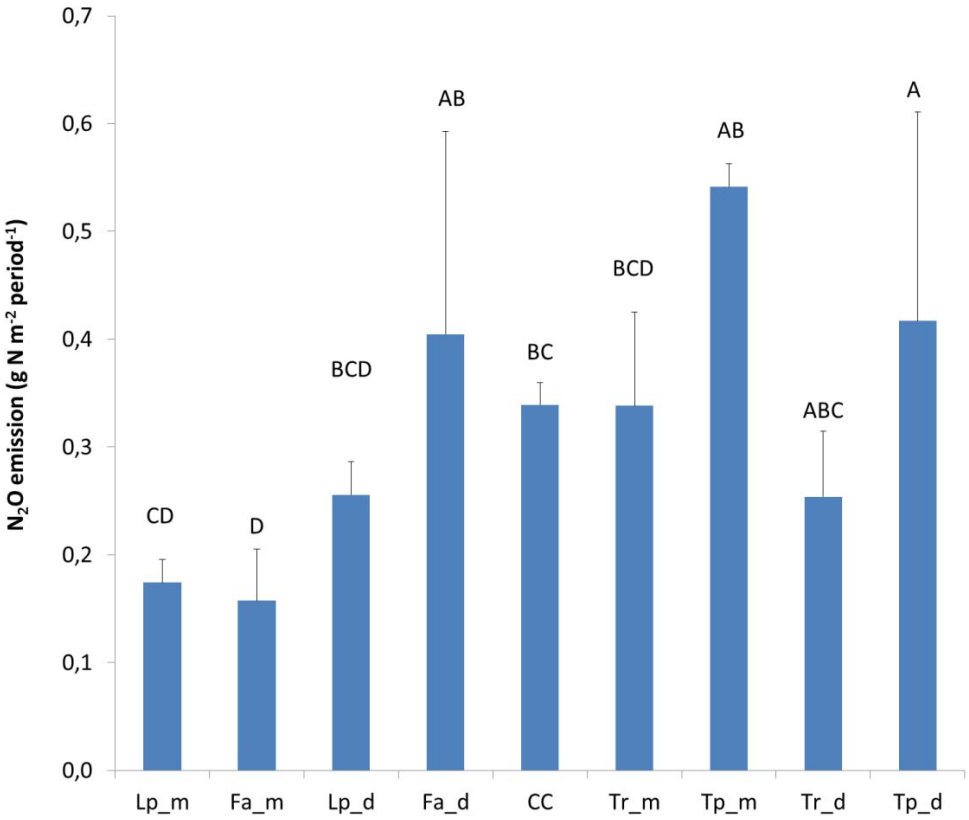


**Figure 10: Cumulative $N_2O$ emission (g N $m^{-2}$) for the 71 days of the experiment (error bars: SE); columns not sharing the same letter are significantly different.**

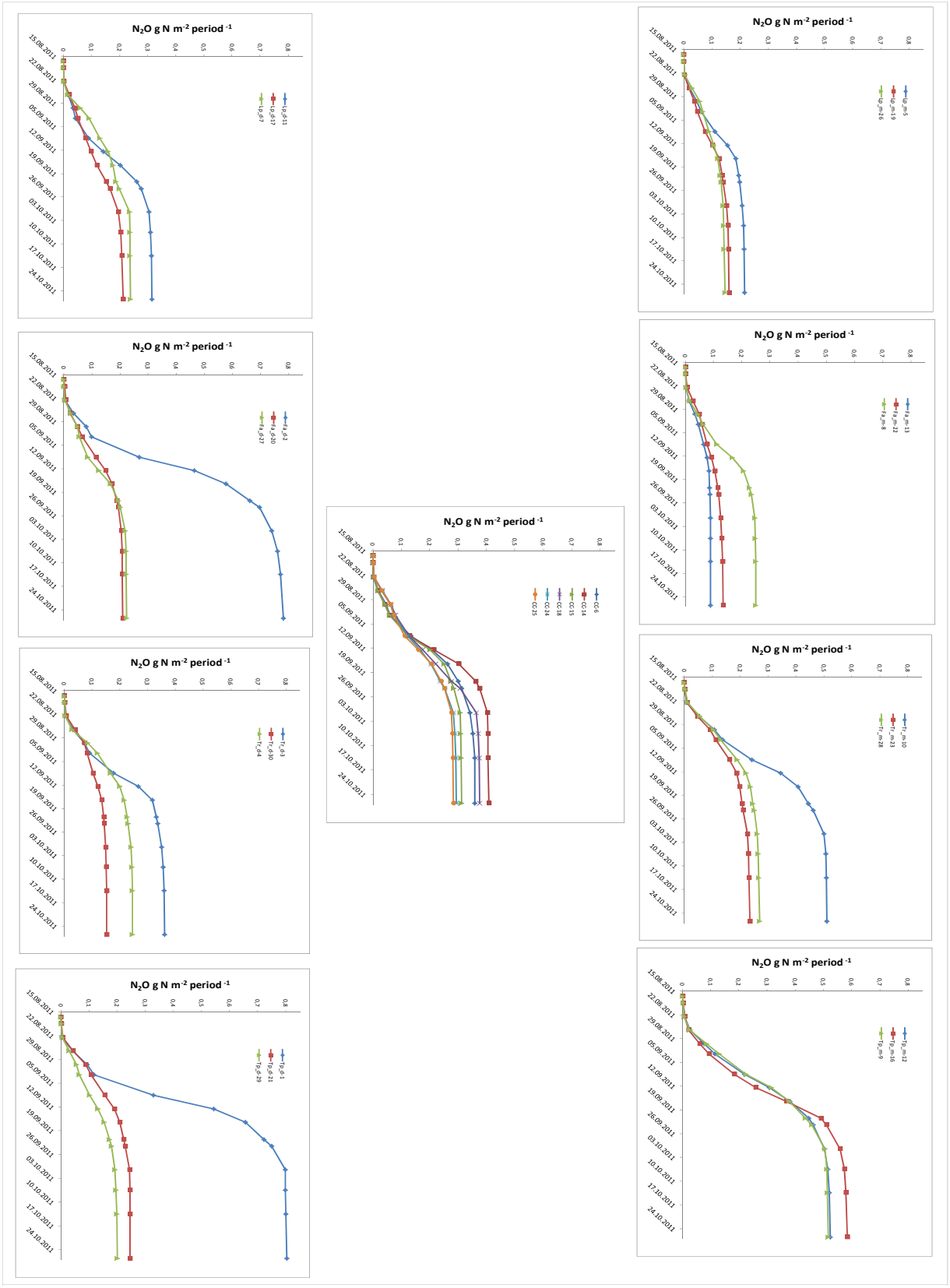


**Figure 11: Cumulative $N_2O$ emissions (g N $m^{-2}$) for single replicates in each treatment**

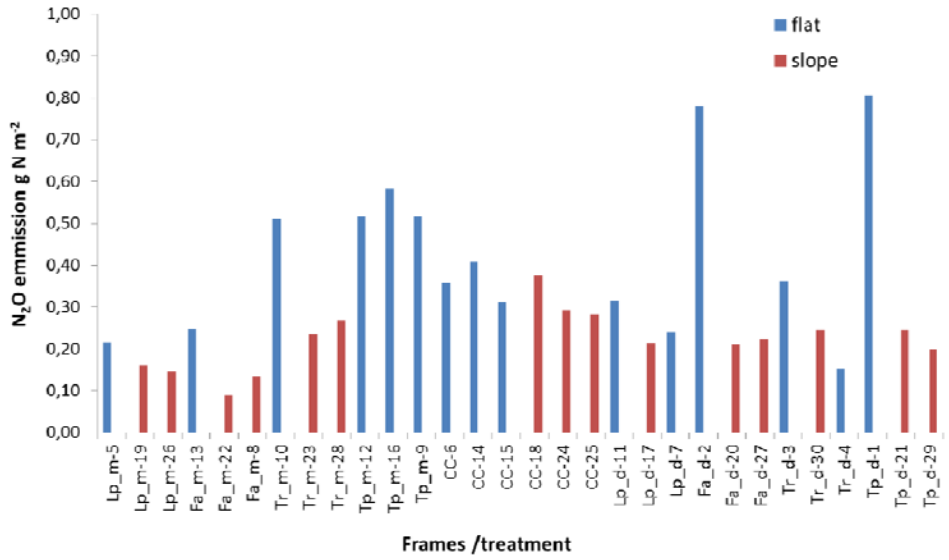


**Figure 12: Spatial variability of cumulative $N_2O$ emission (g N $m^{-2}$) for each frame/treatment. Red bars indicate frames lying on the slope and the blue bars indicate frames on a flat surface.**

## 3.4 Clover Density

A weak positive correlation between red clover percentage and cumulative $N_2O$ emission (g N $m^{-2}$ 71 $days^{-1}$) could be established on the basis single plots ($R^2$= 0.29) and is shown in figure 13. The coefficient of determination was similar when cumulating $N_2O$ emission flux for the period between the application of artificial urine (22/08) and cutting 16/09 ($R^2$= 0.27). No such relationship was found for white clover ($R^2$= 0.011). However, there were two frames (Tp_d 1 and Fa_d 2) that showed exceptionally high $N_2O$ flux rates. When these were removed, the correlation coefficient was $R^2$= 0.50 for all treatments, and $R^2$= 0.57 for red clover alone. Clover percentage was mostly ranging between 10% and 40%. Fig. 13 shows that frames where clover ranged between 37-62% gave relatively low cumulative $N_2O$ emissions (0.15-0.25 g N $m^{-2}$). These frames (fame numbers 17-30; fig. 4) were positioned on the sloping part of the

field. Distinction was made between red and white clover, since the emissions from white clover Tr_m were lower than those from red clover Tp_m.

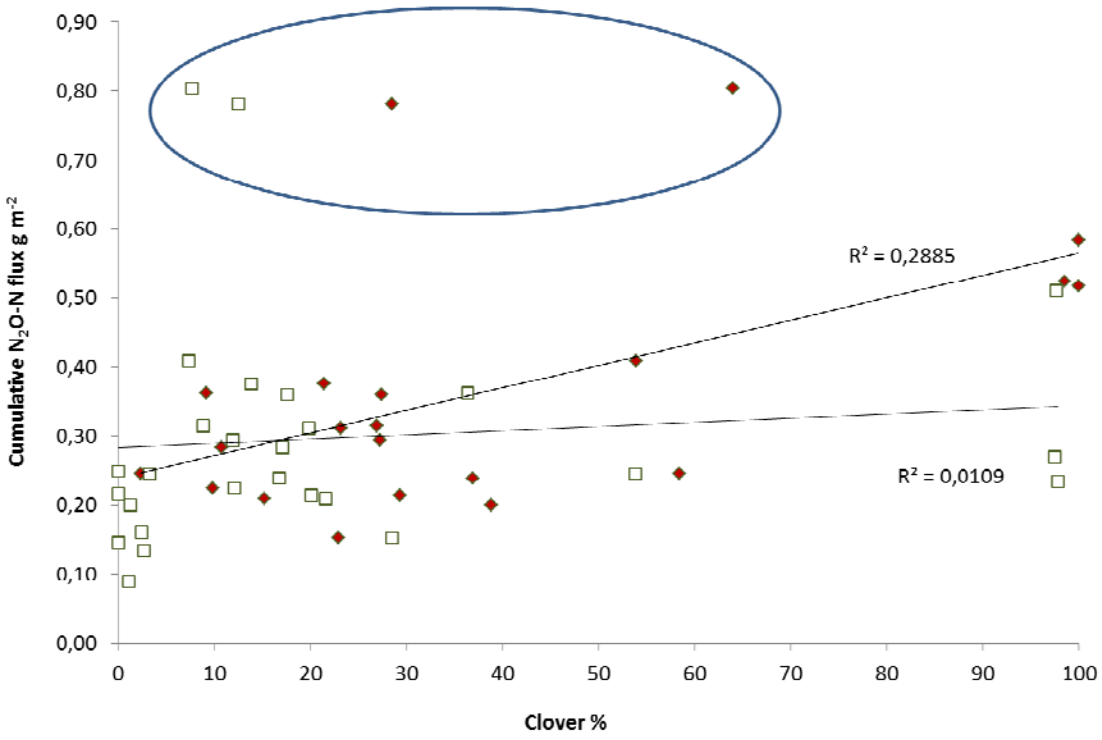


**Figure 13 Relationship between clover density and cumulative $N_2O$ emissions for each plot. Red squares represent red clover, and white squares represent white clover. Replicates with exceptionally high fluxes are highlighted by a circle.**

## 3.5 N yield

Average N yield for the different treatments did not differ significantly from the centroid except for Lp_m and Tr_m (Fig. 14). The highest average N yield among mixed stands was found in Fa_d (11.59 g $m^{-2}$), whereas Fa_m was the highest among pure stands. Yet, using ANOVA test and Fischer method showed that Fa_d was significantly higher than Fa_m (P= 0.006). The lowest average N yield was found in Tr_m (5.67 g $m^{-2}$).

Frames 23, and 28 (97.43 and 78.01 g $m^{-2}$, respectively) of treatment Tr_m had a smaller dry matter yield than frame 10 (143.06 g $m^{-2}$) of the same treatment (see annex). Lowest N yields were found with white clover (Tr_m, 284.26 g N $m^{-2}$ and Tr_d 30, 4.94 g $m^{-2}$). N yield from red clover was slightly higher than from white clover.

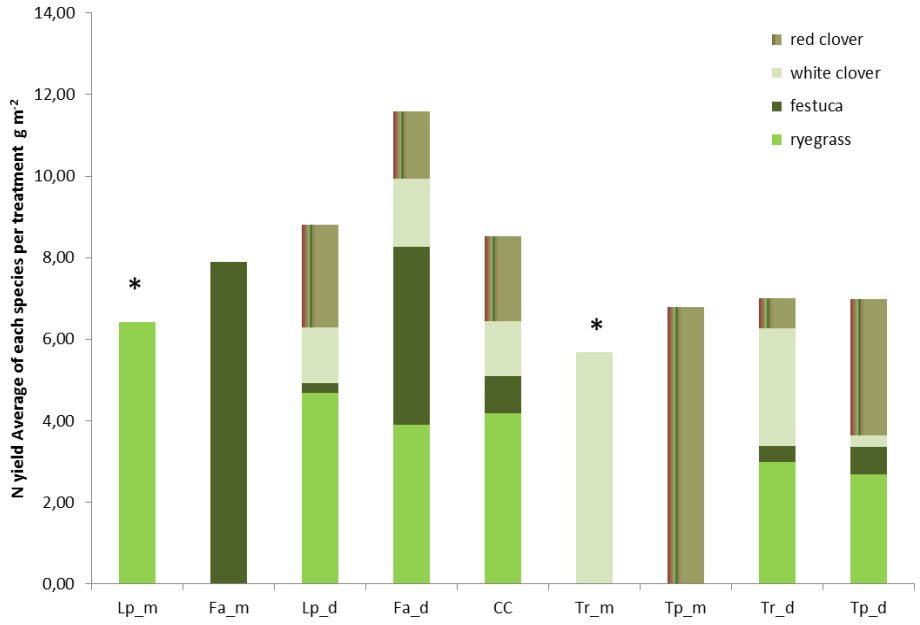


**Figure 14: Average N yield per species for each treatment (g N $m^{-2}$). * denotes treatments that are significantly different from the centroid. P value = 0,001 and df = 9 for dry matter weight and a P value of 0,003 for N yield**

There was a significant negative relationship between N yield in grass (*L. perenne* and *F. arundinacea*) and the percentage of clover. N yield was relatively high with a clover density between 30-50% (Fig 15).

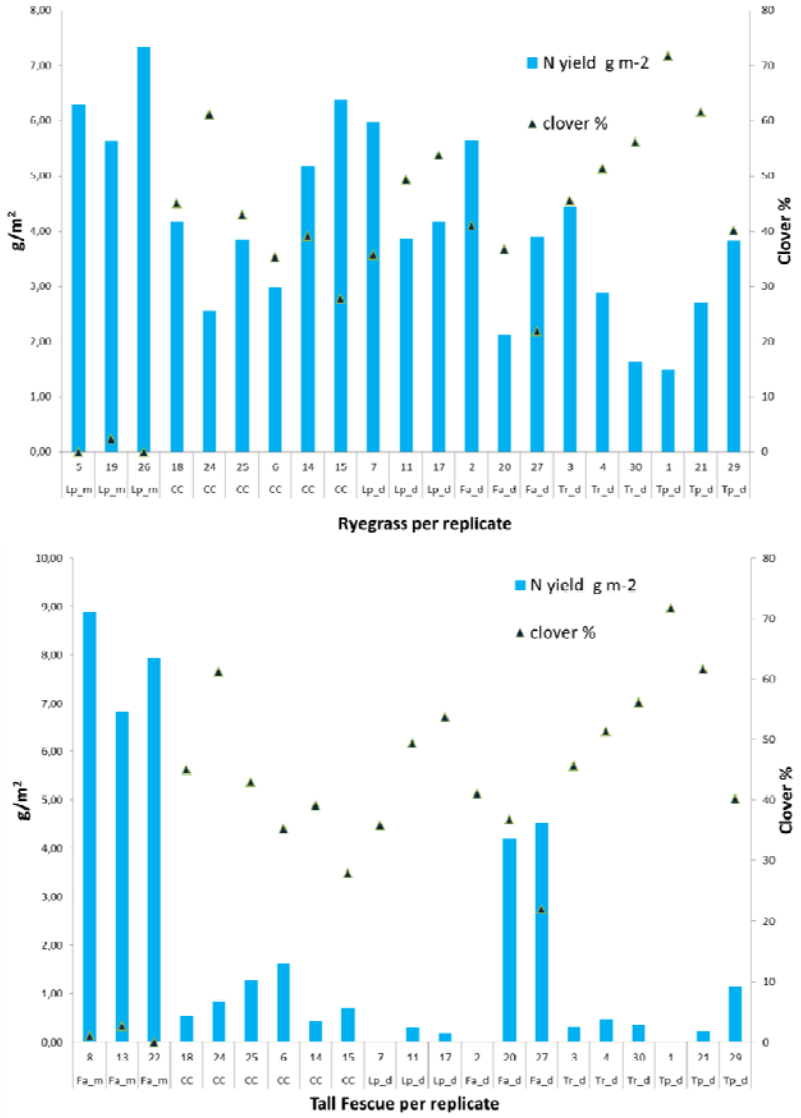


**Figure 15 Relationship between clover percentage and N yield in ryegrass and tall fescue per replicate/treatment. P < 0.05**

## 3.6 $N_2O$ intensity

The ratio between N lost by $N_2O$ emission and N uptake as measured in the harvest of the cut on Sept 13th was calculated to show the amount of $N_2O$ produced per unit N produced. $N_2O$ emission per unit harvested N was lowest in stands dominated by grasses (Lp_m, Fa_m, Lp_d) and tended to be higher in treatments dominated by clover (Tp_d, Tr_m and Tp_m) (Fig. 17). The grass-clover mixture Fa_d and the clover-grass Tr_d showed comparable ratios as the centroid. Tp_m produced the highest average value was 0.08 ± 0.01 g $N_2O$-N per g N yield $m^{-2}$, while dominant red clover gave a value of 0.07 ± 0.06 g $N_2O$-N per g N yield $m^{-2}$. Pure white clover stand Tr_m produced a lower value 0.06 ± 0.01 g $N_2O$-N per g N yield $m^{-2}$.

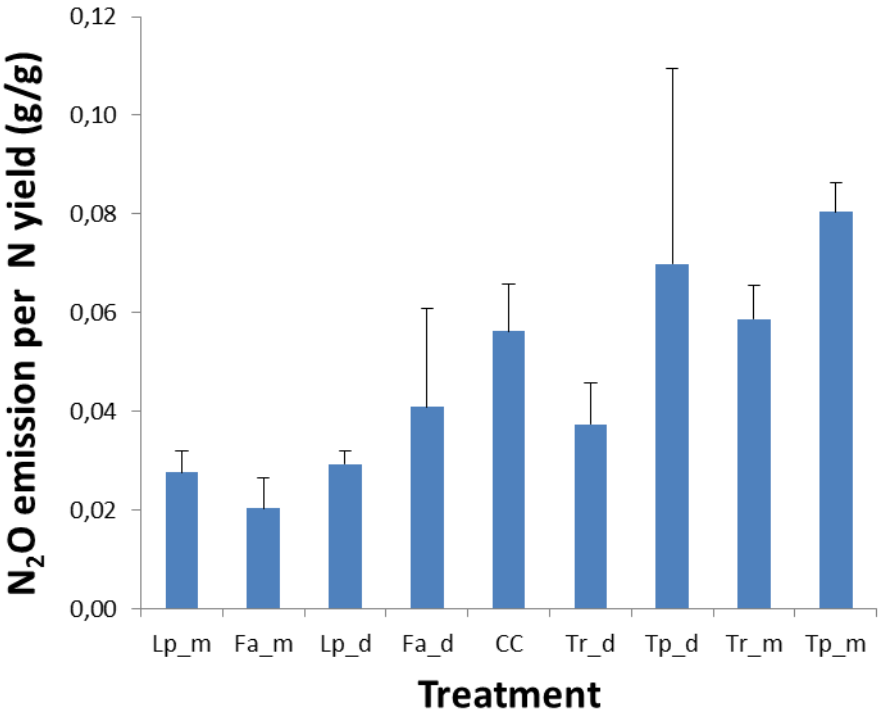


**Figure 16 Cumulative $N_2O$ emission per N yield (g/g) for individual replicates replicate.**

There was a significant relationship between the clover percentage per frame and the values of $N_2O$-N per g N yield $m^{-2}$ (*P*= 0.0004: data not shown). The relationship remained significant even after pure clover treatments were excluded (*P*= 0.004: data not shown).

# 4 Discussion

From an agroecological perspective, this study tackled the N cycle in three subsystems: the soil, the atmosphere and the plant populations, and simulated the influence of a fourth subsystem (grazing livestock) in the form of artificial urine. The nexus of these subsystems was N fixation by clover, and the extent to which the percentage of clover in mixed stand with grass would influence $N_2O$ emission from urine patches. Addition of mineral N, especially in high concentration as in urine is expected to halt N fixation for a certain period, causing the plant population to flip from N fixation to competition for soil mineral N. The increase in plant growth immediately after urine application indicated that a large proportion of urine N or its hydrolysis product $NH_4^+$ was removed by plant uptake. However, observed fluctuations of extractable $NH_4^+$ and $NO_3^-$ in soil indicated that nitrification and denitrification occurred simultaneously, likely fueling high $N_2O$ emission rates observed after artificial urine application. Yet denitrification seemed to be the predominant process for $N_2O$ formation, especially during the second peak of $N_2O$ emissions since $NO_3^-$ content in the soil increased while $NH_4^+$ decreased and rainfalls were abundant (Fig. 7). Unfortunately temporal resolved soil mineral N dynamics for this period are missing, so that nothing can be said about the role of $NO_3^-$ leaching during heavy rainfall events. $NO_3^-$ declined towards the end of the trial, which coincided with a decline in $N_2O$ emissions, supporting the idea that denitrification was the predominant process for $N_2O$ formation during the second half of the experiment.

$N_2O$ emissions in pure red clover stands (0.54 g $m^{-2}$) were remarkably higher than in other treatments, particularly when comparing with pure white clover and dominant white clover. This observation lead to the assumption that red clover has a stronger effect on $N_2O$ emissions than white clover, however, it should be considered that the white clover treatment (Tr_m) showed very variable results among replicates, with the frame Tr_m 10 being higher than frames Tr_m 23 and Tr_m 28 in both $N_2O$ emissions (Fig. 11) and dry matter yield (Annex 1) Red clover dominated plots Tp_m showed the highest soil $NO_3^-$ contents with 6.73 g $m^{-2}$ and 20 cm depth on Sept 30$^{th}$being the

highest concentration observed (Fig. 7C). This is in accordance with the observation of Niklaus, Wardle et al. (2006) who showed that legumes, particularly red clover accumulate nitrates, and increase the abundance of nitrifiers. This offers an explanation to the high $N_2O$ emissions associated with pure red clover stands.

An explanation for reduced dry matter yield and lower $N_2O$ emission in frames Tr_m 23 and 28 may be infestation by slugs which was observed in these plots. Most probably slug infestation led to reduced root biomass and hence limited rhizodeposition. Root exudates are rich in readily degradable carbon which may be a source of $NH_4^+$ for nitrifying bacteria and a source of energy for heterotrophic microorganisms depleting oxygen in the rhizosphere and supporting anoxic metabolism such as denitrification. Topographic position (hill slope versus foot slope) may have been another factor influencing N cycling in the plots. It was striking that the two outliers in cumulative $N_2O$ production (Fa_d2 and Tp_d1) were situated on the flat portion of the experimental field and that plots situated on the slope tended to have lower emissions within each treatment (Fig. 12). This suggests that runoff of mobile $NO_3^-$ by leaching or surface runoff may have occurred during the wet summer, overriding the effect of plant composition of N cycling characteristics and associated $N_2O$ emissions. The high intra-site variability is ultimately also the reason for the low level of significance in differences found between the treatments (Fig. 10).

Towards the end of the experiment, $N_2O$-N losses were lowest and $NO_3^-$ contents highest in the ryegrass/white clover treatment Lp_d (Fig. 9). This is in contrast to Peyraud and Delaby (2006) who attributed high nitrate leaching to high legume percentage and associated higher N fixation. Likewise, $NO_3^-$ was higher in monocultures of clover than in mixed stands of red and white clover treatments towards the end of the trial (Fig. 10) suggesting that the monocultures used mineral N less efficiently than the mixed swards . The opposite was observed for the grass treatments. Here, $NO_3^-$ concentrations were higher in the grass dominated mixed treatments than in the rye grass and tall fescue monocultures (Fig. 10). Since the harvest took place on the 13$^{th}$ of September, the light and temperature were too low for plants to take up soil N

mineralized in the root zone. Although regrowth took place as observed during the gas sampling, Fig. 10 shows that dominant grass mixtures and pure clover could not make use of residual mineral N in autumn as was found in dominant clover mixtures Tr_d and Tp_d.

A significant effect of clover density on $N_2O$ emissions was seen in this study: pure clover/dominant clover treatments produced more $N_2O$ than pure grass treatments, yet there were no significant differences among grass-clover mixtures regarding $N_2O$ emissions even if the frames showing anomalously high emissions were excluded. There was no statistically significant difference between emissions from Lp_m and Lp_d although emissions were higher in the latter. This finding supports the hypothesis that clover can be included in the mixed pasture without increasing $N_2O$ emissions. Galbally, et al. (2010) argued that grass-legume pastures contribute with less than 0.1 g $m^{-2}$ $N_2O$-N based on his own and other studies (e.g. Simek et al. 2004). However, no figures, to the best of my knowledge have been reported on $N_2O$ emissions from urine patches with different clover densities except in the study conducted by Klumpp et al. (2011) which did not include contrasting concentrations (0% to 100%) of clover as in the present study. Yet the period of my study is rather short as compared to other studies, in which treatment effects were more evident.

The difference in the effect of white clover and red clover percentages might be due to the lower competitive capacity of white clover to establish itself in the field in comparison to red clover. However, given the shear amount of ammonium entering the soil-plant system by urea application, similar $N_2O$ emissions could have been expected from clover stands regardless of clover species. Plotting clover percentages against cumulative $N_2O$ emissions showed that the same clover percentages affected cumulative $N_2O$ emission differently (Fig. 13); red clover showed a weak positive relationship between coverage and $N_2O$ emissions, whereas white clover did not. $N_2O$ emissions in red clover dominated treatments were clearly higher than in white clover dominated treatments in the second half of the experiment (Fig. 8), when the initial effect of ammonia hydrolyzing from urea was leveling off. Thus, higher $N_2O$ emissions in red clover stands as compared to white clover stands seem to be associated with less efficient N uptake during the late growing season and/or specifically higher

mineralization/nitrification in the root zone of red clover as compared with white clover.

All in all, it can be concluded that the clover percentage had a limited effect on stimulating $N_2O$ emissions from urine patches in mixed sward pastures, in comparison to other factors such as topography. In recent studies, no long term effects of clover on soil N pools were detected, except for increased $N_2O$ loss from a fertilized low percentage clover treatment (Klumpp et al., 2011).

The role of short term N fixation in inducing $N_2O$ emission has been investigated in recent studies, and was found to play a very limited role for $N_2O$ emissions (Carter and Ambus, 2006).

Following the fluctuation of $N_2O$ emission, rainfall seemed to be the main driver of $N_2O$ emissions. However, there was no significant relationship found between $N_2O$ emissions and WFPS. High WFPS is recurrently found to be an important factor for high $N_2O$ emissions e.g. (Ruzjerez, et al. 1994; Anger, et al. 2003) but environmental factors may not always relate directly to $N_2O$ emissions in statistical analysis (Simek, et al. 2004) likely because denitrification rates as well as the rate of $NO_3^-$ loss by leaching are influenced by the interaction of topography and soil water regime at the landscape level (Pennock, Vankessel et al. 1992).

With regard to N yield, the effect of clover percentage was clearer than for $N_2O$ emissions. There was a significant relationship between clover percentage and N yield, as well as grass N yield (Fig. 15). This indicates that the presence of clover in mixture with grass resulted in an increased uptake of N by grass which should potentially decrease the amount of N available for soil microorganisms and hence affecting $N_2O$ emissions. Scaled for N-yield (Fig. 16), cumulative $N_2O$ emissions throughout the period between urine application and harvest were highest in clover treatments (monoculture and as dominant species in mixture with grass) and despite the reduction in biomass from white clover because of slug infestation, $N_2O$ emissions per unit N yield was also high compared to the grass treatment, yet slightly lower than the other clover treatments. This shows that clover pure stands have higher $N_2O$ emissions per unit N

yield whereas no difference was seen between grass monocultures and grass mixtures. Thus, clover monocultures should be avoided.

The inclusion of clover in mixtures had no effect on area-based $N_2O$ emissions in urine affected pastures (Fig. 10), which is in line with the hypothesis that clover in multispecies swards does not increase $N_2O$ emissions. At the same time, the inclusion clover resulted in an even N yield in companion grass (Fig. 14) as hypothesized.

Biodiversity is an important feature in agroecosystems, it provides a wide array of ecosystem services, in addition to resilience of the whole system in response to disturbance. In the experiment described in my study, urine deposition represents this disturbance, which in grazing pastures is accompanied by both dung and trampling. Understanding the delicate balance between the functional groups in the grass sward, as well as biotic (including macrofauna) and abiotic effects is important for devising adequate management strategies especially in climate neutral farming systems.

In conclusion, clover percentage and species distribution had little effect on urine-associated $N_2O$ emissions. However, treatment effects became evident towards the end of the experiment when the effect of urine had leveled off. For the entire experiment (including post-harvest $N_2O$ emissions), N-yield scaled emissions seemed to be higher with increasing clover percentage, warranting that there might be tradeoff between increasing N uptake by companion grass and N yield-scaled $N_2O$ emissions in grazed multispecies pastures.

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## Annex I: Dry matter and Nitrogen yield per species/frame g $m^{-2}$

| treatment | frame | plot | plant species | Dry mass g $m^{-2}$ | total N % | N yield g $m^{-2}$ | N-yield, per frame g $m^{-2}$ | Average N yield per treatment |
|---|---|---|---|---|---|---|---|---|
| Lp_m | 5 | S10 | ryegrass | 106,36 | 5,91 | 6,28 | 6,28 | |
| Lp_m | 19 | N1 | ryegrass | 98,42 | 5,72 | 5,63 | 5,63 | |
| Lp_m | 26 | N1 | ryegrass | 131,93 | 5,56 | 7,34 | 7,34 | 6,42 |
| Fa_m | 8 | N11 | festuca | 168,79 | 5,26 | 8,88 | 8,88 | |
| Fa_m | 13 | N11 | festuca | 136,87 | 4,99 | 6,83 | 6,83 | |
| Fa_m | 22 | N2 | festuca | 156,67 | 5,07 | 7,95 | 7,95 | 7,89 |
| Tr_m | 10 | N3 | white clover | 143,06 | 5,30 | 7,58 | 7,58 | |
| Tr_m | 23 | N12 | white clover | 97,43 | 5,31 | 5,18 | 5,18 | |
| Tr_m | 28 | N12 | white clover | 78,01 | 5,47 | 4,26 | 4,26 | 5,67 |
| Tp_m | 9 | N13 | red clover | 125,58 | 4,95 | 6,22 | 6,22 | |
| Tp_m | 12 | N13 | red clover | 166,32 | 3,98 | 6,62 | 6,62 | |
| Tp_m | 16 | N13 | red clover | 176,31 | 4,26 | 7,52 | 7,52 | 6,79 |
| CC1 | 18 | N5 | festuca | 12,05 | 4,56 | 0,55 | 6,30 | |
| CC1 | 24 | N5 | festuca | 18,66 | 4,59 | 0,86 | 4,13 | |
| CC1 | 25 | N5 | festuca | 26,45 | 4,86 | 1,28 | 6,88 | |
| CC1 | 18 | N5 | red clover | 47,69 | 4,28 | 2,04 | | |
| CC1 | 24 | N5 | red clover | 101,34 | 4,25 | 4,31 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC1 | 25 | N5 | red clover | 41,84 | 4,13 | 1,73 | | |
| CC1 | 18 | N5 | ryegrass | 83,67 | 4,99 | 4,18 | | |
| CC1 | 24 | N5 | ryegrass | 54,45 | 4,70 | 2,56 | | |
| CC1 | 25 | N5 | ryegrass | 76,76 | 5,01 | 3,85 | | |
| CC1 | 18 | N5 | white clover | 30,66 | 5,13 | 1,57 | | |
| CC1 | 24 | N5 | white clover | 13,76 | 5,17 | 0,71 | | |
| CC1 | 25 | N5 | white clover | 35,83 | 4,87 | 1,75 | | 8,46 |
| CC2 | 6 | N14 | festuca | 30,25 | 5,39 | 1,63 | 5,98 | |
| CC2 | 14 | N14 | festuca | 9,65 | 4,58 | 0,44 | 7,78 | |
| CC2 | 15 | N14 | festuca | 14,06 | 5,08 | 0,71 | 8,06 | |
| CC2 | 6 | N14 | red clover | 27,66 | 5,01 | 1,39 | | |
| CC2 | 14 | N14 | red clover | 48,94 | 4,43 | 2,17 | | |
| CC2 | 15 | N14 | red clover | 20,06 | 4,74 | 0,95 | | |
| CC2 | 6 | N14 | ryegrass | 53,46 | 5,55 | 2,97 | | |
| CC2 | 14 | N14 | ryegrass | 99,78 | 5,18 | 5,17 | | |
| CC2 | 15 | N14 | ryegrass | 120,64 | 5,30 | 6,39 | | |
| CC2 | 6 | N14 | white clover | 17,78 | 6,00 | 1,07 | | |
| CC2 | 14 | N14 | white clover | 21,32 | 5,50 | 1,17 | | |
| CC2 | 15 | N14 | white clover | 31,88 | 5,53 | 1,76 | | 8,61 |
| Lp_d | 7 | N15 | festuca | | | | 9,13 | |
| Lp_d | 11 | N6 | festuca | 6,57 | 4,76 | 0,31 | 8,26 | |
| Lp_d | 17 | N6 | festuca | 3,91 | 4,69 | 0,18 | 8,76 | |
| Lp_d | 7 | N15 | red clover | 47,84 | 4,79 | 2,29 | | |
| Lp_d | 11 | N6 | red clover | 46,55 | 4,98 | 2,32 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lp_d | 17 | N6 | red clover | 62,13 | 4,70 | 2,92 | | |
| Lp_d | 7 | N15 | ryegrass | 111,68 | 5,35 | 5,97 | | |
| Lp_d | 11 | N6 | ryegrass | 73,79 | 5,24 | 3,87 | | |
| Lp_d | 17 | N6 | ryegrass | 73,94 | 5,65 | 4,18 | | |
| Lp_d | 7 | N15 | white clover | 15,69 | 5,53 | 0,87 | | |
| Lp_d | 11 | N6 | white clover | 31,92 | 5,53 | 1,76 | | |
| Lp_d | 17 | N6 | white clover | 28,16 | 5,24 | 1,48 | | 8,80 |
| Fa_d | 2 | S16 | festuca | 12,08 | 4,96 | 0,60 | 10,25 | |
| Fa_d | 20 | N7 | festuca | 83,29 | 5,04 | 4,20 | 10,01 | |
| Fa_d | 27 | N7 | festuca | 95,00 | 4,78 | 4,54 | 10,74 | |
| Fa_d | 2 | S16 | red clover | 56,16 | 4,78 | 2,68 | | |
| Fa_d | 20 | N7 | red clover | 30,28 | 4,58 | 1,39 | | |
| Fa_d | 27 | N7 | red clover | 21,93 | 4,17 | 0,91 | | |
| Fa_d | 2 | S16 | ryegrass | 104,15 | 5,43 | 5,65 | | |
| Fa_d | 20 | N7 | ryegrass | 42,94 | 4,94 | 2,12 | | |
| Fa_d | 27 | N7 | ryegrass | 78,54 | 4,96 | 3,90 | | |
| Fa_d | 2 | S16 | white clover | 24,62 | 5,33 | 1,31 | | |
| Fa_d | 20 | N7 | white clover | 42,94 | 5,37 | 2,31 | | |
| Fa_d | 27 | N7 | white clover | 26,75 | 5,19 | 1,39 | | 10,34 |
| Tr_d | 3 | S8 | festuca | 7,07 | 4,57 | 0,32 | 8,94 | |
| Tr_d | 4 | S17 | festuca | 9,35 | 4,99 | 0,47 | 7,10 | |
| Tr_d | 30 | N8 | festuca | 8,32 | 4,30 | 0,36 | 4,94 | |
| Tr_d | 3 | S8 | red clover | 14,78 | 4,49 | 0,66 | | |
| Tr_d | 4 | S17 | red clover | 30,66 | 4,65 | 1,43 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tr_d | 30 | N8 | red clover | 2,28 | 3,80 | 0,09 | | |
| Tr_d | 3 | S8 | ryegrass | 79,53 | 5,59 | 4,45 | | |
| Tr_d | 4 | S17 | ryegrass | 54,15 | 5,33 | 2,89 | | |
| Tr_d | 30 | N8 | ryegrass | 32,64 | 5,03 | 1,64 | | |
| Tr_d | 3 | S8 | white clover | 58,52 | 5,99 | 3,51 | | |
| Tr_d | 4 | S17 | white clover | 38,23 | 6,07 | 2,32 | | |
| Tr_d | 30 | N8 | white clover | 52,36 | 5,46 | 2,86 | | 6,99 |
| Tp_d | 1 | S9 | festuca | 0,00 | 0 | 0,00 | 5,40 | |
| Tp_d | 21 | N9 | festuca | 4,86 | 4,63 | 0,23 | 7,08 | |
| Tp_d | 29 | N9 | festuca | 23,37 | 4,88 | 1,14 | 7,76 | |
| Tp_d | 1 | S9 | red clover | 76,00 | 4,50 | 3,42 | | |
| Tp_d | 21 | N9 | red clover | 88,42 | 4,39 | 3,88 | | |
| Tp_d | 29 | N9 | red clover | 61,37 | 4,38 | 2,69 | | |
| Tp_d | 1 | S9 | ryegrass | 28,19 | 5,30 | 1,49 | | |
| Tp_d | 21 | N9 | ryegrass | 53,27 | 5,09 | 2,71 | | |
| Tp_d | 29 | N9 | ryegrass | 71,21 | 5,39 | 3,83 | | |
| Tp_d | 1 | S9 | white clover | 9,12 | 5,33 | 0,49 | | |
| Tp_d | 21 | N9 | white clover | 4,86 | 5,28 | 0,26 | | |
| Tp_d | 29 | N9 | white clover | 1,98 | 5,03 | 0,10 | | 6,75 |