Environmental Engineering and Management Journal September 2014, Vol.13, No. 9, 2201-2210
http://omicron.ch.tuiasi.ro/EEMJ/

"Gheorghe Asachi" Technical University of Iasi, Romania

FACTORS CONTROLLING KEY NITROGEN FLUXES IN GRASSLAND ECOSYSTEMS: CASE STUDIES – SOUTHERN ROMANIA

Carmen Postolache*, Florina Botez

University of Bucharest, Department of Systems Ecology and Sustainability, Splaiul Independentei, no. 91-95, 050095, Bucharest, Romania

Abstract

Enhanced nitrogen inputs to both terrestrial and aquatic ecosystems affects biodiversity at all its levels. Grasslands provide a complex range of functions, due to their wide representation throughout terrestrial ecosystems. Numerous studies demonstrate soil acidification, eutrophication and species richness reduction as a result of elevated reactive nitrogen availability. The present study aims to assess soil nitrogen stocks and transformations for two Romanian grasslands during November 2011 – November 2013, integrating the microbial population dynamics of two important functional groups involved in terrestrial nitrogen cycle: ammonifiers and denitrifiers. Our research focused on pristine systems, as well as on an experiment that simulated high nitrogen inputs of 100 kg N/ha/year (during 2012 and 2013) for one of the two selected study areas.

Key words: grassland, microbial processes, mineralization, nitrogen fertilization, nitrogen fluxes

Received: March, 2014; Revised final: August, 2014; Accepted: September, 2014

1. Introduction

Nitrogen is one of the essential macronutrients for biological system, being included in vital biomolecules such as proteins, nucleic acids, chlorophyll, amino sugars, vitamins (Coleman et al., 2004). The Earth's atmosphere is the main reservoir of this element, containing about 78% molecular nitrogen, which is unavailable for organisms in this form. The reactive nitrogen exists in various compounds and oxidation stages, ranging from -3 to +5. Living organisms have an average nitrogen content of about 6.25% dry weight (Bothe et al., 2007).

Although the total amount of nitrogen in terrestrial biomass is relatively small as compared to that in atmosphere (Schlesinger and Bernhardt, 2013), biological systems represent the most active component of its global cycle. Reactive nitrogen derived primary from nitrogen fixation is available to biota in the range of 60-195 TgN/year (Cleveland et al., 1999, cited by Schlesinger and Bernhardt, 2013). In consequence of the rapid uptake of nitrogen by organisms and high mobility of its inorganic ions (especially nitrate), the pool of reactive nitrogen in soil is small. Nitrogen is a limiting factor for plant growth in many terrestrial systems.

The exponential growth of human population led to increased demands for resources. Large amounts of fertilizers have been added to soils in order to improve their fertility and enhance crop production. The intensification of anthropospheric metabolism resulted in the magnification of nitrogen fluxes. Some estimates indicate that industrial production contributes with more than 130 TgN/year to reactive nitrogen fluxes (Schlesinger and Bernhardt, 2013).

Nitrogen biological fixation almost doubled as a consequence of human activities, reaching about 60 TgN/year (Herridge et al., 2008, cited by Schlesinger and Bernhardt, 2013). As a conclusion, the contribution of anthropic activities to nitrogen inputs

* Author to whom all correspondence should be addressed: e-mail: carmen_postolache83@yahoo.com; Phone: +40744495917; Fax: +40213181571

in terrestrial ecosystems exceeds that of natural processes (Vitousek et al., 1997). Some estimations account for an increase of anthropogenic nitrogen fixation rate of about 60% by 2020 (Galloway et al., 2004).

The anthropic emissions of nitrogen mostly consists of reactive nitrogen compounds, of which some are atmospheric pollutants (nitric oxide, nitric dioxide, ammonia) and can enter terrestrial ecosystems via wet and dry depositions. The largest emissions of gaseous nitrogen are from industrial developed countries but due to transboundary nature of atmospheric pollution, nitrogen deposition is a widespread problem. In most European countries (more than 70%) more than a half of the ecosystems are affected by atmospheric nitrogen depositions (Dise and Stevens, 2005). It is estimated that these depositions will further increase with population growth (Tilman et al., 2001).

The spatial distribution of oxidized and reduced nitrogen would vary depending on the point and diffuse emission sources. The largest amount of excessive reactive nitrogen is in the form of ammonia, while about 30% of total nitrogen emissions are the oxidized species (Oliver et al., 1998). Agriculture is the major contributor to ammonia emissions through its volatilization from domestic animal wastes (Bouwman et al., 2002) and the use of fertilizers. Some NH_3 results from catalytic conversion of nitrogen oxides formed during the combustion of fossil fuels in automobiles (Emmenegger et al., 2004). Smaller emissions arise from biological processes in soils (Ganzeveld et al., 2002) and biomass burning (Kaiser et al., 2012).

The increase of reactive nitrogen emissions by human interventions was followed by changes in the pathways, rates and efficiency of nitrogen cycling. In the last 60 years the global nitrogen input to ecosystems has more than doubled (Smil, 2000) and soil fertility was considerably improved. A higher nitrogen input initially leads to an increased net primary production and a rise of carbon sink for vegetation (Schlesinger and Bernhardt, 2013). Direct toxicity of some nitrogen species (ammonia, nitrite) leads to foliar damage, physiological changes and growth limitation (Britton and Fisher, 2010).

Primary productivity and litter production increase with nitrogen availability, but after a certain load, plant diversity decreases favoring nitrophilous species. Grasslands are vulnerable habitats in this respect, as they usually comprise species adapted to low nutrient levels and sensitive to acidification (Sutton et al., 2011). Species composition is also affected by acidification, with a shift towards acid loving plants. Subsequently, faunal diversity is affected by changes in plant communities. A fraction of the nitrogen entering terrestrial ecosystems remains sequestered in the biosphere, especially in agricultural and forest ecosystems, which can store up to 40% excess nitrogen (Stevens et al., 2005), but the excessive supply of this nutrient at large scale could not be retained and recycled locally. Due to its extremely high mobility, reactive nitrogen is lost to ground waters and surface waters through leaching and runoff, effecting water quality. A major threat of reactive nitrogen on soil is acidification, organic matter depletion and biodiversity loss linked to eutrophication (Sutton et al., 2011). The issues of acidification and eutrophication are of great concern at the European level. In this respect, the Nitrate Directive (Council Directive 91/676/EEC) aims to identify areas contributing to nitrogen pollution of ground water and surface water and to control N losses (EC, 2007).

However, changes induced by increased nitrogen deposition are difficult to detect due to large number of interacting factors that control plant community composition and long time scale over which changes occur. Plant diversity decrease was observed within a wide range of nitrogen deposition, between 2-44 kg N/ha/year, but these changes occur over a period of years. As microorganisms' life cycle is shorter than that of superior plants, a much rapid response is to be expected from this biological compartment. The effect of nitrogen on soil microorganisms' diversity as well as effects of changes of soil biodiversity on nitrogen emissions towards other environmental compartments are not fully understood (Butterbach-Bahl et al., 2011, Laslo et al., 2012). Detailed knowledge of processes involved in nitrogen cycling in terrestrial ecosystems is needed to better understand the impact of nitrogen depositions on ecosystems functioning and to predict ecosystems' response to increased levels of reactive nitrogen (Butterbach-Bahl et al., 2011).

The aim of this study is to determine what correlations exist between different levels of nitrogen inputs and soil processes in grassland ecosystems from Southern Romania. The conceptual background consists of an integrated approach of the issue, analyzing the main compartments of a grassland ecosystem. In this first step of the study, changes in soil chemistry rates of nitrogen fluxes and microbiological communities are investigated.

2. Materials and method

2.1. Study area

Two grassland ecosystems were selected using historical nitrogen deposition data provided by the EMEP database, overlapping remote areas isolated from direct human impact from agricultural and industrial activities and roads. Both systems are located in Giurgiu County in Găvanu-Burdea Plain, a sub-unit of the Romanian Plain.

Study area has a temperate climate, mean temperatures range from -3.3°C (winter) to 29.1°C (summer). Volume of precipitation is similar to both grasslands with mean annual values of 700-800 mm. Analysis of soil maps indicate different soil types, chromic luvisols (Bolintin) and fluvi-calcareous fluvisols (Vadu Lat) (FAO-UNESCO soil classification).

2.2. Experimental design

The main compartments analyzed in this study were bulk atmospheric depositions and topsoils. Physical, chemical and microbiological analyses were carried out with a monthly frequency. From each study case five topsoil samples were sampled for analyses and a subsample was field incubated to determine *in situ* mineralization rates. For a better understanding of nitrogen input impact on grassland nitrogen biogeochemical cycle, an experiment of nitrogen addition (NH_4NO_3) was carried out for Vadu Lat grassland, simulating a contribution of 100 kg N/ha/yr. Two plots with an area of 2 m^2 were delimited and one was used as a reference and "fertilized" with distilled water in order to simulate the effect of nitrogen solution used for the fertilized plot.

The experiment consisted of five annually additions of nitrogen, using the same concentration for each month. From each plot three topsoil samples were monthly analyzed for the same parameters used for the topsoils of monitored case studies.

2.3. Bulk deposition monitoring

Bulk deposition (wet and dry deposition) was sampled using an international standard method using an open funnel and a collector buried below ground in order to mitigate the effects of heat and sunlight on the samples (CLRTAP, 2010). For each site a system was installed with the surface of the collecting funnels set at 1 m above ground. Furthermore, the components used were HDPE materials that have no interaction with the sample. Samples were analyzed with a monthly frequency for volume, pH, conductivity and inorganic nitrogen species. A subsample was continuously homogenized with a magnetic stirrer and used to determine conductivity and pH (measured with Hanna 3000 field instrument). The remaining sample was filtered using Whatman 0.45 μm filter paper and the filtered and non-filtered solutions were used for determination of available nitrogen species using spectrophotometric methods.

2.4. Soil sampling and analyzing

Analysis of soils required five sample units for each grassland system and three sample units from the experimental plots that were monthly collected (November 2011 to November 2013, respectively July 2012 – November 2013 for the experimental nitrogen addition). In addition, each sample unit had a corresponding sample that was used for the determination of *in situ* mineralization rates. As a result, there were ten sample units for both grasslands and six sample units for the experimental plots that were bulked for chemical analysis. Samples were transported in a cooling unit to the laboratory within three hours. Each sample was cleared from roots prior to analysis and separated in two parts, one used for determination of available nitrogen species and the other for the mineralization potential.

Field-moist soil samples were prepared for analyses of inorganic nitrogen forms (ammonium, nitrite and nitrate), moisture, pH and organic matter content. Soil pH was measured with glass electrode (soil:water ratio - 1:4) and soil moisture was measured gravimetrically. Determination of nitrogen species required the extraction of approximately 20.00 g of soil using 0.2 M KCl (e.g. Keeney and Nelson, 1987) then analyzed for NO_3^--N, NH_4^+-N and NO_2^--N using a Thermo Helios γ UV-VIS spectrophotometer. Ammonia was determined using indophenols-blue method (Bremner, 1965), NO_2^--N was determined by nitrite diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye (e.g. Moorcroft et al., 2001) and nitrate was quantified by the phenol disulphonic acid method (e.g. Jagessar and Sooknundun, 2011). All data is reported on dry soil weight. Soil organic matter was estimated by incineration of oven-dry soils at 550°C for four hours.

In situ mineralization rate was estimated using the buried bag technique (e.g. Westermann and Crothers, 1980) by incubating approx. 150 g field-moist soil sample in sterile flasks at 0–15 cm depth for circa a month. Available nitrogen species were measured colorimetrically (as above) both at the beginning and the end of the incubation. Mineralization potential was estimated by incubating field moist samples for two weeks at 37°C in an oxygen and light deprived system to allow complete organic nitrogen mineralization (Waring and Bremner, 1964). At the end of the period nitrogen was extracted using 2 M KCl and distilled water was added to attain a concentration of 0.2 M KCl of the extracting solution. In this case, only ammonium nitrogen was determined, because the time frame allowed complete mineralization and reduction of nitrogen species to ammonium.

Soil microbial analyses included the numerical abundance of two important groups in soil nitrogen cycle: ammonifying and denitrifying bacteria. Sample handling was performed in a sterile environment (either flame proximity or laminar flow hood). For each group, population density was estimated using MPN (Most Probable Number) technique, with a three test-tube system. Approximately 1.0000 g of field moist soil was weighted in a sterile tube and 10 ml of sterile distilled water was added. After mechanical homogenization, four serial dilutions with a factor of 10 were produced in order to inoculate specific growth media: peptone water for ammonifying group and Pochon media for denitrifying group. Due to the higher numerical abundance of ammonifiers, the starting dilutions in this case were with a factor of 100. Inoculated media was then incubated for 24 hours at 37°C and the presence of the functional groups was groups was tested using specific reagents:

Nessler reagent for ammonifying bacteria and sulfanilamide in concentrated H_2SO_4, Griess reagents and Nessler for denitrifying bacteria.

3. Results and discussions

A major nitrogen source for natural and semi natural ecosystems is atmospheric deposition. Reactive nitrogen mainly comes from fossil fuel combustion (as NOx) and agriculture (NH3 and related compounds) and can be deposited hundreds of kilometers from emission sources. The distribution of nitrogen deposition reflects the intensity of different anthropic activities. The results of atmospheric deposition monitored in the study areas showed low nitrogen inputs for both sites, with monthly values not exceeding 1 kg N/ha. For the study period, total inorganic nitrogen depositions reached 6.62 kg N/ha for Bolintin and 6.79 kg N/ha for Vadu Lat site. The contribution of different sources is revealed by the ratio of reduced to oxidized nitrogen species (Fig. 1). The reduced form of nitrogen surpassed the oxidized ones in Vadu Lat site during 2012 and was lower than the latter only accidentally between August-October 2013. This can be explained by the predominant contribution of emissions from the breeding farms located in the proximity of Vadu Lat (nearest at 4 km) and of the industrial complex for fertilizer production from Călugăreni (about 35 km). Fertilizer manufacture and application as well as fermented animal food, such as tillage, have an important role in ammonia emissions (Pearson and Stewart, 1993).

Ammonia has a relatively short lifetime, ranging from a few hours to a few days and therefore it deposits rapidly (Oliver et al., 1998). Contrary to information from literature attesting seasonal variation for ammonia, peaking in spring and lower in winter (ApSimon et al., 1987), our monitoring data shows higher concentrations both in winter and spring. Bolintin grassland is characterized by a predominance of oxidized nitrogen forms versus reduced forms with the exception of the period: December 2012 - April 2013. This site is closer to larger urban areas with heavy traffic and industry which can account for the dominance over agricultural emissions.

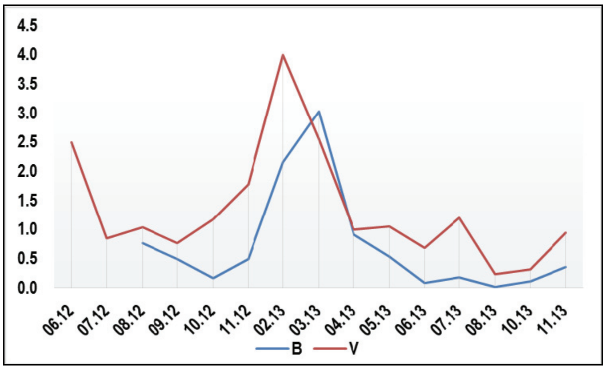

Fig. 1. Reduced versus oxidized nitrogen species ratio from bulk depositions from Bolintin (B) and Vadu Lat (V) during June 2012 – November 2013

The yearly inputs of all inorganic nitrogen forms through bulk depositions are of 5.29 kg N/ha for Bolintin site and 5.02 kg N/ha for Vadu Lat in 2013. These depositions are in the range of 3-10 kg N/ha/year established at the European level as critical loads for grassland ecosystems (Metcalfe et al., 1998), in order to prevent soil nitrogen saturation.

The impact of nitrogen depositions of these ecosystems is influenced by the height of the input, exposure time, soil buffering capacity and land management (Dise and Stevens, 2005). Due to the complexity of these factors at different space scales, grassland ecosystems' sensibility varies (Bleeker et al., 2011). On an ecosystem scale, soils are not only the main reservoir for nitrogen (Davidson, 1994; Batjes, 1996), but also the compartment where most of its transformations occur. Physico-chemical processes like diffusion, leaching, volatilization lead to nitrogen transfer at local and regional scales (Erisman et al., 2008). Nitrogen turnover in soil is affected by soil properties such as texture and clay content. Soils with fine texture retain to a greater extent moisture and organic carbon and become anaerobic more easily after rainfall events. On the other hand, sandy soils favor nitrate leaching. Soil ionic exchange capacity is essential for its buffering capacity which affects nitrogen retention.

Data regarding soil types and traits was obtained by analyzing maps provided by the European Soil Data Center, using Google Earth files (http://esdac.jrc.ec.europa.eu/). Soil taxonomy according to this data is different for the study areas, Bolintin soil is chromic luvisol and Vadu Lat is calcaric fluvisol, which suggests a finer texture of this grassland soil.

Dominant surface textural class supports this statement, with a medium fine texture for Bolintin soil (<35% clay and <15% sand), whereas Vadu Lat soil presents a fine texture (35% < clay < 60%). Both grassland soils exhibit low organic carbon levels (1-2%) and high base saturation (>50%) of topsoils, but differ in regards to soil cationic exchange capacity, which is lower for Bolintin (<15 $cmol^+/kg$) and average for Vadu Lat (15-40 $cmol^+/kg$). Another important information regarding soils from study area is the physical degradation susceptibility - crustification and erodability, which are higher for Bolintin.

Soil pH is influenced by nitrogen deposition and nitrate leaching, both processes leading to acidification. Acidic soils are more sensitive to nitrogen deposition. The investigated sites are characterized by a similar pH dynamics, the fluctuations ranging between 6.19 – 7.76 for Bolintin and 5.74 – 6.85 for Vadu Lat. The slightly lower pH values recorded for Vadu Lat can be explained by a more intense decomposition process of the higher organic matter content. This hypothesis is supported by the results obtained for *in situ* mineralization rates. After circa a month of incubation, all soil samples showed lower pH values compared to initial levels.

Plant nutrient availability is dependent on soil organic matter content and its decomposition rate (Parton et al., 1988). According to literature data (Schlesinger, 2013), nitrogen fixation accounts for less than 20% of the necessary nitrogen for plants, the rest being supplied by soil organic matter decomposition and internal recycling.

Significant differences were observed between the two investigated sites in respect to organic matter content. Elevated levels were observed for Vadu Lat soil samples as a consequence of both higher density of plant cover and fine soil texture (Fig. 2). The mean soil organic matter content in 2012 was 8.89% for Bolintin and 11.51% for Vadu Lat. A slight seasonal variation was perceived, with accumulation tendencies during fall and winter when dead plant material builds up and decomposition rates decline at lower temperatures. The storage and release of reactive nitrogen from organic matter is driven by litter production, its decomposition rates and the balance between these processes. The importance of soil organic matter content in nitrogen cycling was highlighted in a study focused on nitrogen fluxes in a semi-arid grassland ecosystem (Machon et al., 2010). The authors emphasized that organic matter, pH and temperature are the most important factors influencing soil nitrogen flows.

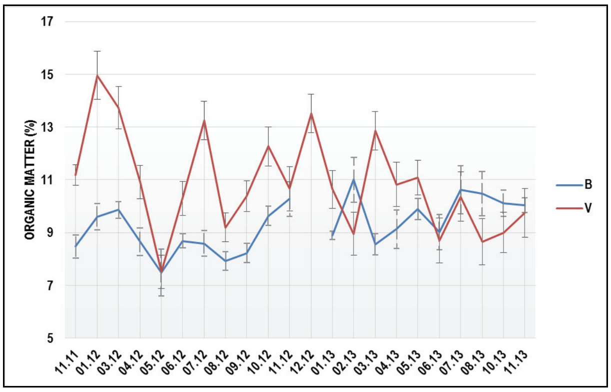

Fig. 2. Soil organic matter content from Bolintin (B) and Vadu Lat (V) during November 2011 – November 2013

One of the assumptions of existing knowledge on nitrogen cycling until 1990s was that plants take only inorganic nitrogen species. Many studies performed in the last 20 years showed that plants are able to take up organic nitrogen as well, in the form of amino acids (Rennenberg et al., 2009). Other studies showed that when nitrogen is not in limited amounts, its organic compounds remain the major source of nutrients for plants (Jackson et al., 2008). Harrison and collaborators showed for British grassland that most plant species preferred inorganic nitrogen compounds over amino acids (Harrison et al., 2007). Therefore we looked to determine whether nitrogen is a limiting factor for plant growth in our study sites and tried to appraise its dynamics in relationship with key cycling processes.

Similar to organic matter dynamics, soil ammonium content showed smaller variations for Bolintin, between 0.05 – 4.25 mg N-NH_4^+/kg dry weight. Wider variation range was observed for ammonium nitrogen from Vadu Lat soil samples, from 0.45 to 7.83 mg N-NH_4^+/kg dry weight. Some studies attested an optimal soil ammonium nitrogen level of 2.5 – 3 mg N-NH_4^+/kg dry weight, higher values being detrimental to plant species richness (Jones and Chapman, 2011). The range of ammonium levels we determined fits this interval for Bolintin grassland, but approximately about two times greater for Vadu Lat. Same pattern was observed for nitrate, reaching a maximum of 16.50 mg N-NO_3^-/kg dry weight in Vadu Lat and only 8.99 mg N-NO_3^-/kg dry weight for Bolintin, while nitrite was about one order of magnitude lower in both sites. As a consequence, dynamics of available inorganic nitrogen for plant uptake is characterized by much higher levels for Vadu Lat and numerous fluctuations, with a slight tendency of accumulation during fall (Fig. 3).

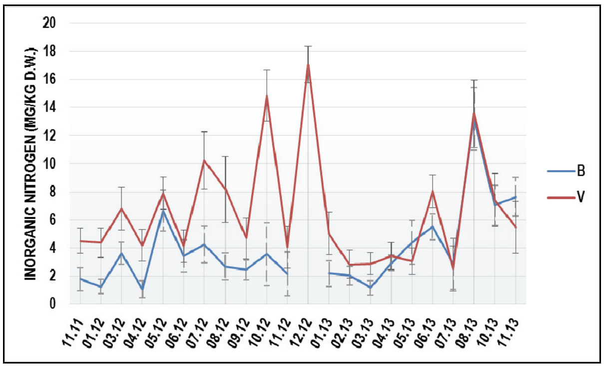

Fig. 3. Soil inorganic nitrogen (mg/kg dry weight) from Bolintin (B) and Vadu Lat (V) during November 2011 – November 2013

Trying to explain these dynamics, we have to stress out that there are many driving factors which can affect the conversion between reduced and oxidized nitrogen species, therefore the availability of soil inorganic nitrogen varies considerably among different ecosystems. The most important processes interfering in soil inorganic nitrogen dynamics are plant uptake, immobilization and transformations mediated by microorganisms (mineralization, nitrification, ammonification and denitrification), leaching, absorption to clay particles (Johnson et al., 2000). At any given time, the loads of extractable inorganic nitrogen compounds are the results of all these processes.

The higher nitrate levels as compared to ammonium observed for Vadu Lat suggests that this nutrient is not a limiting factor for primary production, situation in which ammonium would be prevailing, as literature data indicates (Jackson et al., 1989; Stark and Hart, 1997). We also must mention that plant preference for either nitrogen species depends on plant identity and abiotic factors (temperature, pH). When both reduced and oxidized nitrogen occur with similar levels, most plants prefer ammonium. There are studies showing that this nitrogen species implies lower energy costs (Butterbach-Bahl et al., 2011). This case is presumably happening for Bolintin grassland, where the two nitrogen species are comparable.

Apart from plant uptake, a key role in nitrogen retention and loss was attributed to ratios between mineralization and ammonium immobilization, as well as between nitrification and nitrate microbial immobilization (Stockdale et al., 2002). These ratios were used as indicator of potential nitrogen loss and nitrogen retention capacity of ecosystems (Dannenmann et al., 2006). The results of *in situ* mineralization rates indicate a more intense process for Vadu Lat site as compared to Bolintin (Fig. 4).

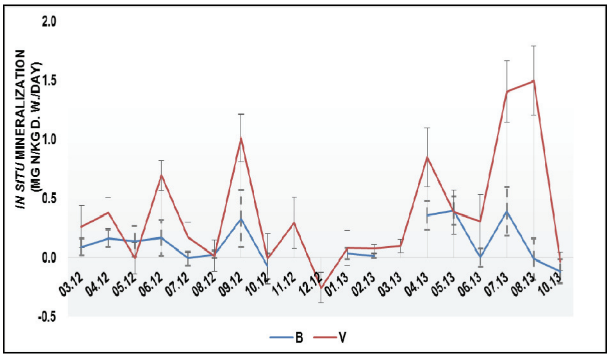

Fig. 4. *In situ* mineralization rates (mg N/kg dry weight/day) from Bolintin (B) and Vadu Lat (V) during November 2011 – October 2013

This can be explained by the richer soil organic matter content for this site, which is the substrate of ammonification process. The dependency of mineralization on soil organic matter content but also on the carbon bioavailability is widely reported in literature (Accoe et al., 2004; Booth et al., 2005; Perakis and Sinkhom, 2011). Few negative values were obtained for both sites meaning that after one month of *in situ* soil incubation the content of mineral nitrogen was smaller than the initial levels, which indicates nitrogen gaseous loss. Losses of nitrogen can occur during the experiment because incubation depletes oxygen availability, favoring denitrification. Mean values of net mineralization rates were 0.12 mg N/kg dry weight soil/day for Bolintin and 0.38 mg N/kg dry weight soil/day for Vadu Lat grassland. The variability of this process is very high even at an ecosystem level due to the complexity of interacting factors that govern it, as it can be easily observed from the plot in Fig. 4. A simple computation of the yearly topsoil net mineralization based on the determined mean rate provided us a rough estimation of 70 kg N/ha/year, which is in accordance with levels mentioned Hatch et al. (1990) as typical for grasslands, ranging from 40 to 90 kg N/ha/year.

Quantification of soil samples mineralization potential highlighted the importance of microbial communities in performing this process. Much higher rates of mineralization potential were obtained for Vadu Lat ecosystem (Fig. 5).

In order to quantify the contribution of microbial communities on nitrogen cycling we proceeded to assess population sizes of ammonifiers and denitrifiers during the study period. Experimental data analysis showed big fluctuations of ammonifiers' density for both grasslands, between 100 – 12000 individuals/g d.w. soil. Although there are significant differences of soil organic matter content for both studied sites, which is the ammonifiers' substrate the ranges of population densities and their dynamics are similar. This suggests that the driving forces of mineralization process are more complex, including substrate availability, temperature, humidity. A deeper data analysis revealed a good correlation between soil ammonium levels and ammonifiers' population size for both grasslands (Fig. 6), indicating that ammonium acts like a regulating factor of this microbial group. The effect is similar with that of an inhibitor of a biological process.

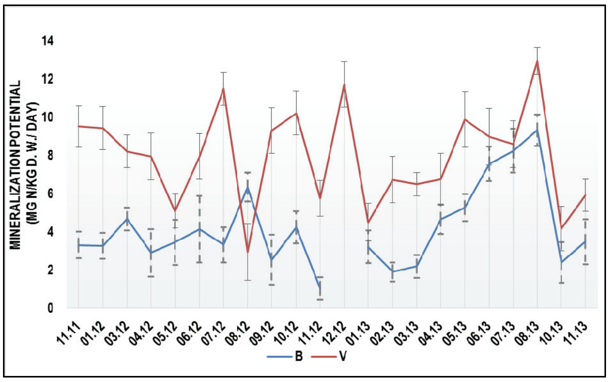

Fig. 5. Mineralization potential rates (mg N/kg dry weight/day) from Bolintin (B) and Vadu Lat (V) during November 2011 – November 2013

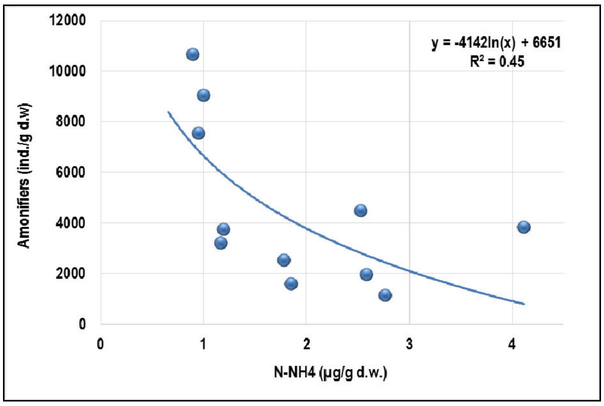

Fig. 6. Ammonifiers' density dependency on ammonium-nitrogen from Bolintin (B) during April 2012 – November 2013

Positive correlation was obtained between mineralization potential rate and microbial population size. A plateau is reached at higher densities, indicating that the process is dependent on substrate quantity and accessibility (Fig. 7).

A paradigm which dominated the understanding of nitrogen cycles until 1990s asserted that mineralization is the limiting step of nitrogen cycling. The new paradigm takes into account two steps of mineralization process: depolymerization of organic macromolecules to smaller, bioavailable molecules (dissolved organic nitrogen), followed by ammonification of bioavailable organic nitrogen to ammonium (Jackson et al., 2008). In this new view, the rate determining step is the depolymerization process, which is carried out by fungal and bacterial enzymes (Jackson et al., 2008). Both depolymerizing and ammonifying microbes are carbon limited, which sustains the hypothesis we inferred for the plateau reaching and also illustrates carbon and nitrogen cycles coupling.

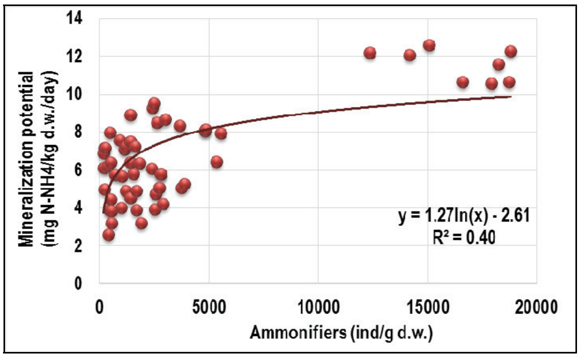

Fig. 7. Mineralization potential (mg N-NH4+/kg dry weight/day) dependency on ammonifiers' density (individuals/g dry weight) for Vadu Lat during April 2012 – November 2013

As we already stressed out, *in situ* mineralization is partially influenced by the overlap with denitrification process. The end result is the decrease of soil inorganic nitrogen content and a decline of mineralization rate is to be expected alongside denitrification intensification. This was confirmed by the correlation we obtained between *in situ* mineralization and denitrifiers' population size (Fig. 8).

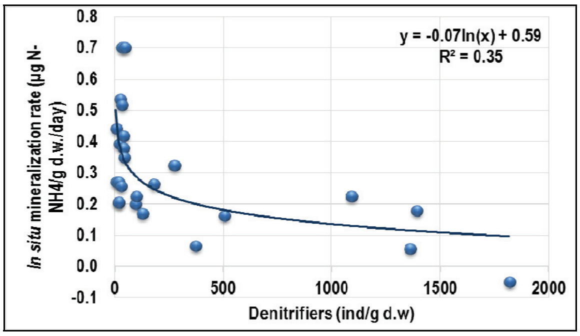

Fig. 8. *In situ* mineralization rate (mg N-NH4+/kg dry weight/day) dependency on denitrifiers' density (individuals/g dry weight) for Bolintin during April 2012 – November 2013

In order to complete gathered information through field measurements of soil nitrogen stocks and fluxes, an experiment of supplementary nitrogen fertilization was performed on Vadu Lat grassland. The intention was to perceive answers of ecosystem biodiversity components to increased nitrogen inputs. To better discriminate the occurred changes as a result of enhanced reactive nitrogen bioavailability, the level of added nitrogen was 100 kg N/ha/year, previously mentioned in literature as a limit above which detectable changes of bioavailable nitrogen stocks were observed (Bai et al., 2010). Same monitoring program was applied for the reference and fertilized plot as for the sites under investigation. First obtained response was a slight soil acidification, from a mean pH value of 6.33 for the reference plot (V0) to 6.08 for the fertilized one (V10). Similar resulted were mentioned by Zhang et al. (2012).

Soil organic matter content decreased as a consequence of increased nitrogen input, the decline being more significant after the fertilization ceased (Fig. 9). Over the period of the experiment, about 15% organic matter decrease was recorded. The negative effect of nitrogen depositions on carbon sequestration was also reported by other scientists (e.g. Pilkington et al., 2005).

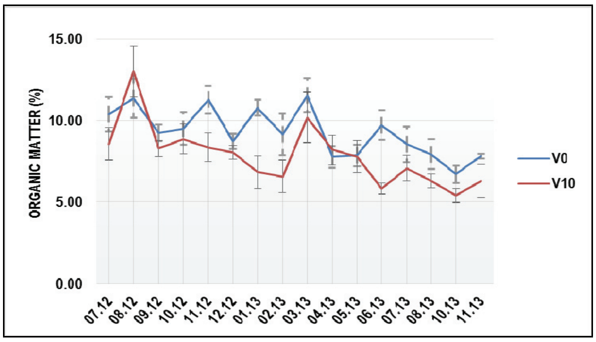

Fig. 9. Soil organic matter content from reference plot (V0) and fertilized plot (V10) during July 2012 – November 2013

A clear tendency of inorganic nitrogen accumulation was observed for the fertilized plot, particularly in the second period of the experiment (Fig. 10). Both reduced and oxidized nitrogen forms increased, from 2.53 mg N/kg to 7.03 mg N/kg soil for N-NH4+ and from 3.90 mg N/kg to 11.83 mg N/kg for N-NO3-. Nitrogen excess decrease was shown to be a slow process (Bobbink et al., 2010).

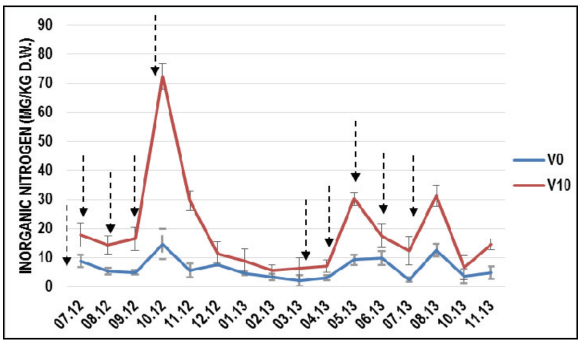

Fig. 10. Soil inorganic nitrogen (mg N/kg dry weight) from reference plot (V0) and fertilized plot (V10) during July 2012 – November 2013; dash arrows represent the months in which fertilizer was applied

Changes in soil organic matter content and available inorganic nitrogen induced modifications at microbial communities' scale, as they have been stressed out by other researchers (e.g. Johnson et al., 2005). The decrease of ammonifiers' density was observed in response to fertilization. This finding supports our previous assumption that ammonium, the end product of ammonification process, has a regulating effect on microbial population (Table 1).

Contrary to the dynamics of this group, the denitrifiers' density increased over three times in the fertilized area (Table 1). These changes were better observed during fertilization months, indicating an immediate response of microbial communities to increased nitrogen inputs. Stimulation of population densities of denitrifiers after nitrogen addition was also noticed by Avrahami et al. (2002), as a result of higher levels of available nitrate, the substrate of denitrification process.

Table 1. Mean microbial population densities of ammonifiers and denitrifiers from experimental plots: reference (V0) and fertilized (V10) during July 2012 – November 2013

Group	***Ammonifiers (ind./g dry weight)***		***Denitrifiers (ind./g dry weight)***	
Plot / Date (mm.yy)	***V0***	***V10***	***V0***	***V10***
07.12	5216	**969**	98	**175**
08.12	5124	**5306**	18	**61**
09.12	5837	**3747**	47	**336**
10.12	543	**1967**	50	**239**
11.12	322	**324**	10	**38**
12.12	5353	**1608**	4	**7**
01.13	2316	**569**	60	**27**
02.13	7458	**4592**	44	**21**
03.13	16565	**11123**	76	**434**
04.13	462	**1615**	79	**93**
05.13	4092	**929**	10	**60**
06.13	16527	**7065**	22	**114**
07.13	9038	**7488**	38	**232**
08.13	744	**1835**	34	**182**
10.13	6575	**360**	122	**373**
11.13	10804	**972**	182	**38**

In situ denitrification process is affected by numerous "factors, being higher in nitrogen - fertilized soils and soils with increased nitrate availability, but is difficult to predict denitrification rates based on our current understanding" (Groffman et al., 2009). It was concluded that plot studies are still needed to improve our knowledge in this respect (Butterbach-Bahl et al., 2011).

Additional fertilization induced an intensification of *in situ* mineralization, which is resulted in the observed decline of soil organic matter (Postolache et al., unpublished data). Similar findings were reported in a grassland ecosystem after fertilization with 80 kg N/ha/year (Zhang et al., 2012). The increased nitrogen input induces complex changes in soil stocks and fluxes of this element. Regulation of nitrogen cycling occurs at different space and time scales and implies microbial activities to a great extent. Our experiment clearly shows rapid changes of microbial communities and nitrogen fluxes mediated by specific functional groups under enhanced inputs of this nutrient.

4. Conclusions

Our research on selected grassland ecosystems tried to answer several questions:

- What changes occur over soil nitrogen stocks of grassland ecosystems under different nitrogen inputs?
- How are key nitrogen fluxes influenced by biotic and abiotic factors?
- What are the responses of microbial communities to experimental addition of reactive nitrogen?

The results of the monitoring program revealed the importance of local hydrogeomorphological characteristics upon nitrogen species stock and dynamics, as well as on its transformation rates.

Mineralization process is more intense for Vadu Lat site, which has a higher organic matter content and mineralization potential, and lower denitrifiers' density. An inhibitory effect of ammonium on the mineralization rate and ammonifiers was observed. Good correlations were obtained between mineralization process and population densities of ammonifiers and denitrifiers.

Increase of reactive nitrogen stocks was noticed as a consequence of higher nitrogen inputs. Changes were observed in soil organic matter content and rate of its mineralization. Microbial communities were differently affected by fertilization, consisting in a growth of population densities of denitrifiers and decrease of ammonifiers. Microbial communities proved to be indicator of enhanced nitrogen inputs due to a short time response and good sensitivity at changes of nitrogen levels.

Plant cover requires an extended time scale in order to respond to changes of available nitrogen amounts and further investigations will be completed in this regard.

Acknowledgements

This work has benefited from the support of the European Social Fund, project POSDRU 107/1.5/S/80765, Human Resources Sectorial Operational Program 2007−2013, priority axis 1, major domain of intervention 1.5. The authors would like to give special thanks to people that worked with us to obtained data for this study: Chifiriuc Carmen, Botos Zamfira, Csolti Andreea and Stanciu Adina.

References

Accoe F., Boeckx P., Busschaert J., Hofman G., Van Cleemput O., (2004), Gross N transformation rates and net N mineralization rates related to the C and N contents of soil organic matter fractions in grassland soils of different age, *Soil Biology & Biochemistry*, **36**, 2075–2087.

ApSimon H.M., Kruse M., Bell J.N.B., (1987), Ammonia emissions and their role in acid deposition, *Atmospheric Environment,* **21**, 1939-1946.

Avrahami S., Conrad R., Braker G., (2002), Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers, *Applied and Environmental Microbiology*, **68**, 5685–5692.

Bai Y., Wu J., Clark C.M., Naeem S., Pan Q., Huang J., Zhang L., Han X., (2010), Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from inner Mongolia Grasslands, *Global Change Biology,* **16**, 358–372.

Batjes N.H., (1996), Total carbon and nitrogen stocks in the soils of the world, *European Journal of Soil Science*, **47**, 151–163.

Bleeker A., Hicks W.K., Dentener F., Galloway J., Erisman J.W., (2011), N deposition as a threat to the World's protected areas under the Convention on Biological Diversity, *Environmental Pollution,* **159**, 2280-2288.

Bobbink R., Hicks K., Galloway J., Spranger T., Alkemade R., Ashmore M., Bustamante M., Cinderby C., Davidson E., Dentener F., Emmett B., Erisman J.W., Fenn M., Gilliam F., Nordin A., Pardo L., de Vries W., (2010), Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis, *Ecological Applications,* **20**, 30-59.

Booth M.S., Stark J.M., Rastetter E., (2005), Controls on nitrogen cycling in terrestrial ecosystems: A synthetic analysis of literature data, *Ecological Monographs,* **75**, 139–157.

Bothe H., Ferguson S.J., Newton W.E. (Eds.), (2007), *Biology of the Nitrogen Cycle*, Elsevier B.V., Amsterdam.

Bouwman A.F., van Vuuren D.P., Derwent R.G., Posch M., (2002), A global analysis of acidification and eutrophication of terrestrial ecosystems, *Water, Air and Soil Pollution,* **141**, 349-382.

Bremner J.M., (1965), *Inorganic forms of nitrogen*, In: *Methods of Soil Analysis, Part 2*, In: *Agronomy* Black C.A. (Ed.), Vol. **9**, American Society of Agronomy 1179-1237.

Britton A.J., Fisher J.M., (2010), Terricolous alpine lichens are sensitive to both load and concentration of applied nitrogen and have potential as bioindicators of nitrogen deposition, *Environmental Pollution,* **158**, 1296–1302.

Butterbach-Bahl K., Gundersen P., Ambus P., Augustin J., Beier C., Boeckx P., Dannenmann M., Gimeno B.S., Ibrom A., Kiese R., Kitzler B., Rees R.M., Smith K.A., Stevens C., Vesala T., Zechmeister-Boltenstern S., (2011), *Nitrogen Processes in Terrestrial Ecosystems*, In: *European Nitrogen Assessment. Sources, Effects and Policy Perspectives*, Sutton M.A., Howard C.M., Erisman J.W., Billen G., Bleeker A., Grennfelt P., van Grinsven H., Grizzetti B. (Eds.), Cambridge University Press, Cambridge-New York, 99-125.

Cleveland C.C., Townsend A.R., Schimel D.S., Fisher H., Howarth R.W., Hedin L.O., Perakis S.S., Latty E.F., Von Fischer J.C., Elseroad A., Wasson M.F., (1999), Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems, *Global Biogeochemical Cycles,* **13**, 623–645.

CLRTAP, (2010), *Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests, Part XIV, Sampling and Analysis of Deposition*, International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests, UNECE ICP Forests.

Coleman D.C., Crossley D.R. Jr., Hendrix P.F., (2004), *Fundamentals of Soil Ecology*, Elsevier Academic Press.

Dannenmann M., Gasche R., Ledebuhr A., Papen H., (2006), Effects of forest management on soil N cycling in beech forests stocking on calcareous soils, *Plant and Soil*, **287**, 279 –300.

Davidson E.A., (1994), *Climate Change and Soil Microbial Processes: Secondary Effects Are Hypothesized From Better Known Interacting Primary Effects*, In: *Soil Response to Climate Change*, Rounsevell M.D.A., Loveland P.J. (Eds.), Springer, Berlin, 156–168.

Dise N.B., Stevens C.J., (2005), Nitrogen deposition and reduction of terrestrial biodiversity: Evidence from temperate grasslands, *Science in China Series C-Life Science*, **48**, 720–728.

EC, (2007), Report from the Commission to the Council and the European Parliament. On implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources for the period 2000–2003, Brussels, 19.3.2007, COM(2007) 120 final, On line at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52007DC0120&from=EN.

Emmenegger L., Mohn J., Sigrist M., Marinov D., Steinemann U., Zumsteg F., Meier M., (2004), Measurement of ammonia emissions using various techniques in a comparative tunnel study, *International Journal of Environment and Pollution,* **22**, 326–341.

Erisman J.W., Sutton M.A., Galloway J.N., Klimont Z., Winiwarter W., (2008), How a century of ammonia synthesis changed the world, *Nature Geoscience*, **1**, 636 –639.

Galloway J.N., Dentener F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S.P., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michaels A.F., Porter J.H., Townsend A.R., Vorosmarty C.J., (2004), Nitrogen cycles: past, present, and future, *Biogeochemistry,* **70**, 153–226.

Ganzeveld L.N., Lelieveld J., Dentener F.J., Krol M.C., Bouwman A.J., Roelofs G.J., (2002), Global soil-biogenic NOx emissions and the role of canopy processes, *Journal of Geophysical Research-Atmospheres,* **107**.

Groffman P.M., Hardy J.P., Fisk M.C., Fahey T.J., Driscoll C.T., (2009), Climate variation and soil carbon and nitrogen cycling processes in a northern hardwood forest, *Ecosystems,* **12**, 927–943.

Harrison K.A., Bol R., Bardgett R.D., (2007), Preferences for different nitrogen forms by coexisting plant species and soil microbes, *Ecology*, **88**, 989-999.

Hatch D.J., Jarvis S.C., Philipps L., (1990), Field measurement of nitrogen mineralization using soil core incubation and acetylene inhibition of nitrification, *Plant and Soil,* **124**, 97–107.

Herridge D.F., Peoples M.B., Boddey R.M., (2008), Global inputs of biological nitrogen fixation in agricultural systems, *Plant and Soil,* **311**, 1–18.

Jackson L.E., Schimel J.P., Firestone M.K., (1989), Short-term partitioning of ammonium and nitrate between plants and microbes in an annual grassland, *Soil Biology & Biochemistry,* **21**, 409–415.

Jackson L.E., Burger M., Cavagnaro T.R., (2008), Roots, nitrogen transformations and ecosystem services, *Plant Biology*, **59**, 341–363.

Jagessar R.C., Sooknundun L., (2011), Determination of nitrate anion in waste water from nine selected areas of coastal Guyana via a spectrophotometric method, *International Journal of Research and Reviews in Applied Sciences* (*IJRRAS),* **7**, 203-212.

Johnson D.W., Cheng W., Burke I.C., (2000), Biotic and abiotic nitrogen retention in a variety of forest soils, *Soil Science Society of America Journal,* **64**, 1503–1514.

Johnson D., Leake J.R., Read D.J., (2005), Liming and nitrogen fertilization affects phosphatase activities, microbial biomass and mycorrhizal colonisation in upland grassland, *Plant and Soil,* **271**, 157–164.

Jones R.O., Chapman S.K., (2011), The roles of biotic resistance and nitrogen deposition in regulating non-native understory plant diversity, *Plant Soil,* **345**, 257–269.

Kaiser J.W., Heil A., Andreae M.O., Benedetti A., Chubarova N., Jones L., Morcrette J.-J., Razinger M., Schultz M.G., Suttie M., van der Werf G.R., (2012), Biomass burning emissions estimated with a global fire assimilation system based on observed fire radioactive power, *Biogeosciences,* **9**, 527–554.

Keeney D.R., Nelson D.W., (1987), *Nitrogen--Inorganic Forms, sec. 33-3, Extraction of Exchangeable Ammonium, Nitrate, and Nitrite*, In: *Methods of Soil Analysis: Part 2, Chemical and Microbiological Properties*, Page A.L. (Ed.), Agronomy, A Series of Monographs, no.9 pt.2, Soil Science Society of America, Madison, Wisconsin USA, 648-649.

Laslo, É., György, É., Gyöngyvér, M., Szentes, S., Salamon, R. V., András, C. D., Szabolcs, L., (2012), The management of N and P nutrition of plants using nitrogen fixing and phosphorus solubilizing bacteria, *Environmental Engineering & Management Journal,* **11**, 371-375.

Machon A., Horvath L., Weidinger T., Grosz B., Pinter K., Tuba Z., Fuhrer E., (2010), Estimation of net nitrogen flux between the atmosphere and a semi-natural grassland ecosystem in Hungary, *European Journal of Soil Science*, October 2010, **61**, 631–639.

Metcalfe S.E., Derwent R.G., Whyatt J.D., Dyke H., (1998), Nitrogen deposition and strategies for the control of acidification and eutrophication across Great Britain, *Water, Air and Soil Pollution*, **107**, 121-145.

Moorcroft M.J., Davis J., Compton R.G., (2001), Detection and determination of nitrate and nitrite: a review, *Talanta,* **54**, 785-803.

Oliver J.G.J., Bouwman A.F., Van der Hoek K.W., Berdowski J.J.M., (1998), Global air emission inventories for anthropogenic sources of NOx, NH_3 and N_2O in 1990, *Environmental Pollution,* **102**, 13148.

Parton W.J., Stewart J.W.B., Cole C.V., (1988), Dynamics of C, N, P, and S in grassland soils: A model, *Biogeochemistry,* **5**, 109-131.

Pearson J., Stewart G.R., (1993), The deposition of atmospheric ammonia and its effects on plants, *New Phytologist*, **125**, 283 –305.

Perakis S.S., Sinkhorn E.R., (2011), Biogeochemistry of a temperate forest nitrogen gradient, *Ecology,* **92**, 1481–1491.

Pilkington M.G., Caporn S.J.M., Carroll J.A., Cresswell N., Lee J.A., Emmett B.A., Johnson D., (2005), Effects of increased deposition of atmospheric nitrogen on an upland Calluna moor: N and P transformations, *Environmental Pollution,* **135**, 469–480.

Rennenberg H., Dannenmann M., Gessler A., Kreuzwieser J., Simon J., Papen H., (2009), Nitrogen balance in forest soils: nutritional limitation of plants under climate change stresses, *Plant Biology*, **11**, 4 –23.

Schlesinger W.H., Bernhardt E.S., (2013), *Biogeochemistry: An Analysis of Global Change* (3rd Edition), Academic Press.

Smil V., (2000), Phosphorus in the environment: Natural flows and human interferences, *Annual Review of Energy and the Environment,* **25**, 53–88.

Stark J.M., Hart S.C., (1997), High rates of nitrification and nitrate turnover in undisturbed coniferous forests, *Nature,* **385**, 61–64.

Stevens W.B., Hoeft R.G., Mulvaney R.L., (2005), Fate of nitrogen-15 in a long-term nitrogen rate study: II. Nitrogen uptake efficiency, *Agronomy Journal,* **97**, 1046–1053.

Stockdale E.A., Hatch D.J., Murphy D.V., Ledgard S.F., Watson C.J., (2002), Verifying the nitrification to immobilisation ratio (N/I) as a key determinant of potential nitrate loss in grassland and arable soils, *Agronomie*, **22**, 831 –838.

Sutton M.A., Howard C.M., Erisman J.W., Billen G., Bleeker A., Grennfeld P., van Grinsven H., Grizzetti B. (Eds.), (2011), *European Nitrogen Assessment*, Cambridge University Press.

Vitousek P.M., Abe, J.D., Tilman D.G., (1997), Human alteration of the global nitrogen cycle: sources and consequences, *Ecological Applications,* **7**, 737–752.

Waring S.A., Bremner J.M., (1964), Ammonium production in soil under waterlogged conditions as an index of nitrogen availability, *Nature,* **201**, 951–952.

Westermann D.T., Crothers S.E., (1980), Measuring soil nitrogen mineralization under field conditions, *Agronomy Journal,* **72**, 1009-1012.

Zhang X., Wang Q., Gilliam F.S., Bai W., Han X., Li L., (2012), Effect of nitrogen fertilization on net nitrogen mineralization in a grassland soil, northern China, Blackwell Publishing Ltd., *Grass and Forage Science*, **67**, 219–230.