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Microbial Responses to Nitrogen Addition in Grassland Soils from Southern Romania

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Abstract: Land use change impact is a local phenomenon with a global impact. A key component in soil nutrient cycling is the microbial compartment that metabolizes dead organic matter and converts detritus into bioavailable nutrients, thus sustaining soil productivity. Soil health and responses induced by increased inorganic nitrogen availability can be better understood if it encompass microbial communities' responses to pressures. Our study focused on two major microbial functional groups, ammonifying and denitrifying bacteria, and their responses to increased nitrogen input in a low human-impact grassland. We used two levels of nitrogen addition (5 g N/year and 10 g N/year) in a two-year study. Our monitoring program included the following soil parameters: humidity, organic matter content (SOM), pH, ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$) stocks, microbial densities of ammonifying and denitrifying bacteria, and soil mineralization potential rates. Soil nitrogen stocks revealed short-term accumulation (nitrate) and longer build-up (ammonium). SOM proved to be paramount in regulating the distribution and density of microbial communities. Positive correlations between microbial density and SOM were obtained. The higher addition significantly impacted these trends, especially on ammonifying bacteria. Mineralization potential rates further confirmed that the main driver for the microbial community is substrate availability.

Keywords: soil nitrogen cycle; grassland; soil microbiota; nitrogen fertilization.

1. Introduction

The increasing needs for resources and services caused by the exponentially growing human population have altered the natural capital. All those have determined the conversion of natural ecosystems and the intensification of agricultural practices to obtain enough livestock and crops to sustain the development of socio-economic systems. According to Vitousek et al., 1997, one of the most important nutrient cycles in terrestrial ecosystems - the nitrogen cycle, has been severely altered by human activities [1]. This led to more than doubling the nitrogen input of the terrestrial nitrogen cycle by fertilizer use, with some estimates suggesting the total anthopic input is ten times higher than a century ago [2]. The most important processes involved are nitrogen fixation, mineralization, nitrification, and denitrification. Large amounts of fertilizers are influencing these processes, and the negative environmental impact is still being addressed to shift to more sustainable agricultural practices [3-5].

The nitrogen cycle in terrestrial ecosystems is controlled and modified by a series of factors that are complex and yet not particularly well understood. There are still limits in understanding the stocks and flows in both carbon and nitrogen cycles [6].

Microorganisms have a particularly important role in soil nutrient cycling; therefore, the interest in microbial communities has exponentially peaked [7-10]. They help reestablish the function and biodiversity in ecosystems. It is known that the functional capabilities of soil microorganisms change across biomes, but the progress in understanding how this is happening is limited. Therefore, there is an increasing need for further studies to understand the diversity and function of microorganisms along with different terrestrial biomes [11,12].

Microbial diversity is critical to ecosystem functioning due to the diversity of processes, such as decomposition, nutrient cycling, soil aggregation, and pathogenicity. According to a current estimate, 1 g of soil may contain up to 10 billion bacteria of possibly 4000–7000 different species and a biomass density of 30−30,000 kg/ha [13]. The exploration of soil microbial communities has just begun, despite the fact they are responsible for lots of soil processes and represent a major pool of living biomass in terrestrial ecosystems. Measurements of the microbial community indicate the status of the ecosystem at a certain point. The information obtained is in relation to management practices and different interventions and manipulation practices. The microbial community could be used as an indicator of the status of the system or the enhancement of the recovery rate of a damaged system [14].

Soil microorganisms respond rapidly to stressors by adjusting activity rates, biomass, and community structure. They are subjected to seasonal fluctuations in environmental conditions such as temperature, water content, pH, and nutrient availability. An important issue to elucidate is how environmental changes and seasonal variations influence qualitative variation in community composition [15]. By analyzing the distribution of bacteria at microhabitat levels, Torsvik and Øvreås found that in soils subjected to different fertilization treatments, more than 80% of the bacteria were located in micropores of stable soil micro-aggregates. Such microhabitats offer the most favorable conditions for microbial growth concerning water and substrate availability. Microbial diversity and community structure are impacted by bulk pH and the type and amount of organic compound input.

Changes in nitrogen availability affect and are affected by the soil microbial community. For example, deposition-driven increases in ammonium and nitrate represent larger substrate pools for nitrification and denitrification, affecting nitrogen loss to aquatic and atmospheric realms. Nitrogen from depositional processes and the fraction that fuels primary production are intimately linked to the underlying soil microbial community [16].

Thus, research should focus on quantifying the available nitrogen species in the soil and identifying the microorganisms responsible for these processes in the soil ecosystems [17]. All those changes at a microscopic scale are important because they determine modifications at greater scales. The results and implications of those changes can be observed in the biogeochemical nitrogen cycle [18]. Combining soil microbiological estimates seems to be relevant for evaluating soil quality [19].

Our study aimed to assess changes in microbial communities after nitrogen addition in a low anthropic impact grassland ecosystem situated in the southern part of the Romanian Plain. The scientific questions are i) which are the changes of soil nitrogen stocks induced by ammonium nitrate fertilization; ii) how rates of some nitrogen transformation processes were influenced by nitrogen addition; iii) microbial responses of functional groups involved in nitrogen cycling to nitrogen fertilization.

2. Materials and Methods

2.1. Study area and research design.

The research site is located in a long-term socio-ecological Romanian research area (LTSER Neajlov basin), the southern part of the country, characterized by the continental climate, near the Vadu Lat locality in Giurgiu County (44°20’’’41.7’41.7“‘‘N 25°’40’34 “E). The research program started in the summer of 2014 and ended in the winter 2015. We selected a low human-impact grassland to observe better the changes induced by increased nitrogen availability via fertilization. Grassland ecosystems provide important ecosystem functions such as carbon sequestration [20], and this biome has been intensively converted to agricultural systems.

The grassland site was selected considering some criteria that allowed the exclusion of several factors with potential impact on the soil nitrogen cycle. Firstly, the grassland area was not impacted by agricultural practices for at least 10 years. The human impact was minimal, the distance from roads being at least 100 m (to avoid nitrogen deposition from transport), and the location was also away from livestock areas (local ammonia emissions). To provide a representative area, the surface was 300 $m^{2,}$ and the soil was relatively homogeneous. The experimental design included three fertilization plots, with an area of 2 m^2, situated at least 5 m apart to avoid cross-contamination. Two plots were fertilized with different levels of nitrogen addition, and the other was blankly fertilized (0 nitrogen addition, using distilled water only, to maintain the same hydrological conditions). The amounts of total N inputs were 0 for the reference plot (V0), 5 g N/m^2/year (V5), and 10 g N/m^2/year (V10).

Sampling was performed in triplicates from the topsoil horizon (0-10 cm depth), which is the most active in terms of physicochemical and microbiological processes. The values presented are the average of those replicates, while the correlations included each individual pair of values for the triplicates. To adequately detect changes in nitrogen’s biogeochemical cycle in soil, the monitoring frequency was fixed at monthly intervals. Soil sampling at the site level was made in compliance with the standards imposed by the selected methods. The following soil parameters were determined: soil pH, moisture, organic matter content, the concentration of ammonia nitrogen ($N\text{-}NH_4^+$), nitrate-nitrogen ($N\text{-}NO_3^-$), nitrite nitrogen ($N\text{-}NO_2^-$), and mineralization potential. We monitored two key microbial functional groups involved in soil nitrogen’s biogeochemical cycle: ammonifying bacteria and denitrifying bacteria.

2.2. Laboratory and field methods.

Soil samples were stored and transported in a cooler after field sampling and prepared for analysis on the same day. Soil pH was determined using a laboratory pH-meter WTW 3000. Water content was determined gravimetrically [21], and soil organic matter was obtained by combustion at 550°C [22].

Available inorganic nitrogen species were determined using standardized spectrophotometry methods by extraction with potassium chloride solution (KCl) 0.2 M [23]. One of the most used methods of spectrophotometric determination of ammonia nitrogen is based on making a compound like an indophenol according to Berthelot, 1859, [24], respectively catalyzed reaction between ammonium with a phenolic compound and a chlorine donor agent, at basic range pH. Determination of nitrate-nitrogen is based on the procedure using phenol-disulphonic acid. This method involves nitration of salicylic acid in acid pH (provided by concentrated sulphuric acid), the resulting compound being yellow in a basic medium (sodium hydroxide) [25,26].

Assessing the mineralization potential involved creating optimal conditions for the decomposition processes of organic matter in mineral compounds. To achieve the maximum potential of microbial ammonifying community, soil samples were maintained for a sufficient period of time (two weeks) to transform the organic content into mineral compounds, providing special conditions such as an anaerobic environment, protection from light, and optimal temperature (about 37°- 40°C) [27].

Microbial densities were estimated using the serial dilutions method – Most Probable Number (MPN) technique, and we used a three serial dilution scheme (with a factor of 10) [28]. The dilutions were inoculated with a specific growth medium: peptone water for ammonifying bacteria and Pochon medium for denitrifying bacteria. After a 48-hour incubation period, the bacterial growth of these groups was tested using reagents that gave a positive reaction with microbial by-products [29]. The results were interpreted using the McCrady table.

3. Results and Discussion

3.1. Soil physico-chemical parameters.

All the processes developed by specific groups of bacteria are impacted by the values of the soil's physical and chemical properties [30]. The processes involved in soil nitrogen cycling are highly complex, so we focused only on the dynamics of some key parameters: soil moisture, pH, organic matter content, and soil bioavailable nitrogen species.

As can be observed from Figure 1, there are small differences between the mean values of soil moisture between the selected plots, which substantiates a good pedological homogeneity. The variations of soil moisture are according to the seasons: higher values in winter and spring when there are more precipitations and lower values in summer and autumn due to evaporation and higher temperatures. This parameter is strongly influenced by the precipitation periods and amounts specific to each season.

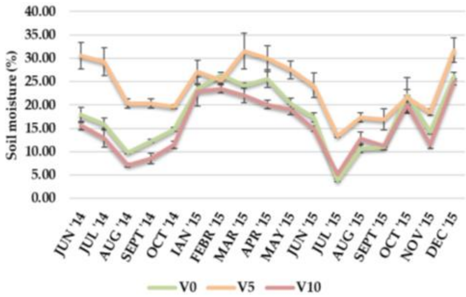

Figure 1. Soil moisture (%) for Vadu Lat site plots (June 2014 – December 2015).

Soil pH represents a key factor influencing soil processes and their chemical, physical, and biological properties [31]. Mean seasonal values obtained for soil samples of the study area are indicated in Table 1. Our study revealed a slightly acidic reaction of soil for all plots and a seasonal variation, with mean values ranging from 5.14 – 6.04 for V0, 5.24 – 6.11 for V5, and 4.64 – 5.94 for V10. The pH values are lower in the plot fertilized with a higher amount of nitrogen input. Soil acidification is a common response to increased nitrogen availability [32-34].

Table 1. Soil pH from Vadu Lat site – June 2014 – December 2015.

	SOIL pH		
Season	**V0**	**V5**	**V10**
Summer 2014	5.89	5.83	5.58
Autumn 2014	5.63	5.24	5.25
Winter 2014	6.04	5.93	5.77
Spring 2015	5.89	6.11	5.94
Summer 2015	5.74	5.76	5.44
Autumn 2015	5.14	5.40	4.64

Another monitored key parameter is soil organic matter, which is important for understanding the global carbon cycle and long-term fertility. The observed differences between the plots were due to variations in vegetation cover density, which was higher in plot V5 (Figure 2). In addition, a seasonal variation is observed in all the plots due to vegetation succession, which influences organic matter turnover.

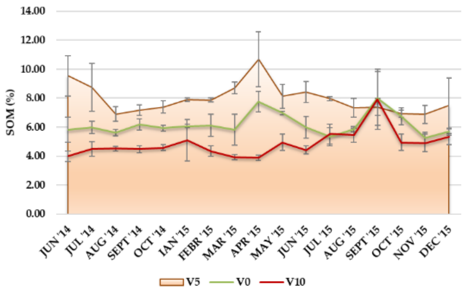

Figure 2. Soil organic matter content (%) from Vadu Lat site plots (June 2014 – December 2015).

Soil microorganisms, mainly fungi and bacteria, are primarily responsible for transforming organic molecules in soil, and their activity is thus a key factor in soil organic matter dynamics. Soil microorganisms decompose litter and soil organic matter in terrestrial ecosystems, which can regulate multiple input and loss pathways of soil carbon and nitrogen. It has been suggested that land-use change can affect the microbial decomposition of litter and soil organic matter, which in turn regulates soil carbon and nitrogen balance in terrestrial ecosystems [35,36].

3.2. Soil bioavailable nitrogen species.

During the decomposition process, organic matter is transformed into more simple compounds, available for plants and microorganisms. The microorganisms can further degrade those substances to form ammonium ions – through the ammonification process. Ammonium ions can be oxidized to nitrate/ nitrite through nitrification [37]. Soil organic matter influences the ammonium and nitrate/nitrite bioavailability for consumers.

Plants take up mainly inorganic nitrogen compounds from soil, ammonium in detriment to nitrate, to cover their needs in terms of nitrogen for growth and development. However, when nitrogen is not a limiting factor, inorganic forms of nitrogen remain the main source of nutrients for plant organisms [38,39]. Ammonium represents the main nitrogen compound that can be uptake by plants and microorganisms because of the lower energetic cost [37].

Soil ammonium levels vary seasonally during our study period, between 0.39 – 18.21 µg N-NH_4^+/g dry weight for V site, 0.73 – 9.24 µg N-NH_4^+/g dry weight for V0 site, 0.26 – 16.90 µg N-NH_4^+/g dry weight for V5 site, and 0.29 – 72.51 µg N-NH_4^+/g dry weight for V10 site.

As observed in Figure 3, the soil ammonium levels are higher in the sites with supplemented nitrogen inputs. These trends can be explained by an increased nitrogen stock via fertilization with ammonium nitrate completed at the end of the summer season. Those values are also influenced by soil organic matter. The system was more resilient in the second year of the experiment, with lower accumulation tendencies. These lower levels also support the theory that ammonium is preferred in plant uptake. After the cold season, increased ammonium concentration was observed for all study areas. This is probably due to the high water content in the soil, which determined low oxygen concentrations, favoring the presence of nitrogen in its reduced form, ammonium ion.

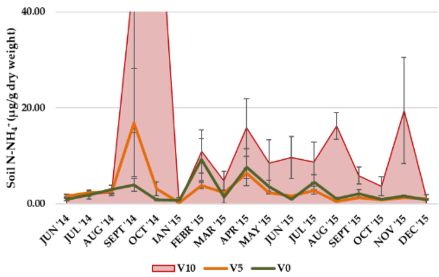

Figure 3. Soil N-NH4$^+$ (µg/g dry weight) from Vadu Lat site plots (June 2014 – December 2015).

Nitrate is easily lost from soils through percolation and has lower concentrations than ammonium, even if the addition ratio is 1:1 N-NO_3^-: N-NH_4^+. Van Breemen et al. (2002) concluded that approximately 23% of the reactive nitrogen from regional systems is exported to rivers and streams [40]. Fertilization with nitrates led to only a temporary accumulation of this chemical form of nitrogen (Figure 4).

Similar conclusions are reported in the literature. Hodge et al. (2000) used ^{15}N-pool-dilution techniques and found much information about N-pool fluxes [41]. For example, ^{15}N-labelling of the NH_4^+ and NO_3^- pools of grassland soil revealed that, even though the NH_4^+ pool was always moderately large, it was extremely dynamic and had a turnover time of ~1 day. The NO_3^- pool was even more dynamic, being consumed as rapidly as it was produced.

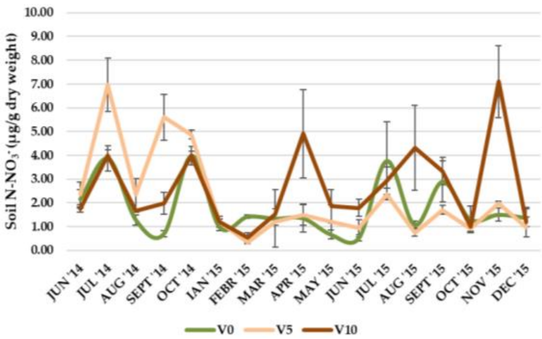

Figure 4. Soil N-NO_3^- (µg/g dry weight) from Vadu Lat site plots (June 2014 – December 2015).

3.3. Soil microbial activity.

The stocks of available nitrogen species result from interaction between microbial processes involved in biogeochemical cycles. The organic matter decomposition process is crucial in any nutrient cycle. This conversion is carried out by microbes and other soil organisms that release or mineralize nutrients as a by-product of their consumption of detritus [33,42].

Nitrogen mineralization varies with soil type and is a measure of soil quality: soils with high N mineralization potential tend to be inherently fertile, while soils with low N mineralization potential tend to be less fertile and require greater agricultural inputs [43]. Traditionally, ammonium has been viewed as the immediate product of mineralization, and often, mineralization is referred to as ammonification. The mineralization potential rates represent the maximum capacity of the microbial community to convert complex compounds to simpler forms and represent a valuable tool in assessing decomposition rates and microbial community efficiency.

Mineralization potential rates we determined for the selected plots vary between 1-8 µg $N\text{-}NH_4^+$/g dry soil/day. These values are similar to those previously reported in the literature for natural and semi-natural grasslands, of 0.32–7.09 µg N g/day [44]. This range further attests that mineralization and immobilization processes are widely influenced by many factors. Increased rates were obtained for V5 plot (Figure 5), which is characterized by a higher density of vegetation and levels of organic matter in the soil. A seasonal dynamic, more evident for V5 plot, can be observed, with enhanced rates from fall to spring, when detritus accumulated on soil and water content was above 7%. Significant activity often occurs at extremes of both temperature and moisture. When soil moisture and temperature are favorable, large organic matter inputs might lead to high rates of microbial activity and the potential for high mineralization and immobilization rates. The quantity and quality of detrital inputs are the main factors that control the rates and patterns of mineralization and immobilization in soil water [42,45].

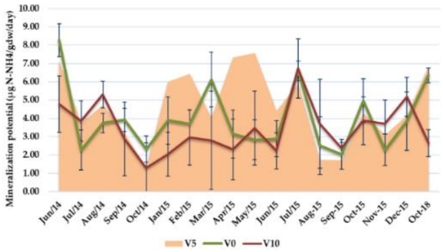

Figure 5. Mineralization potential rates (µg $N\text{-}NH_4^+$/g dry weight/day) from Vadu Lat site plots (June 2014 – December 2015).

We expect e microbial members of soil communities to be very sensitive and rapid indicators of perturbations of land-use changes. They contribute substantially to the resistance and resilience of ecosystems to abiotic disturbance and stress [35]. While assessing the ammonifying functional group, we found a complex dynamic for all plots over the period of investigation (Figure 6). More evident is the seasonal dynamic of ammonifiers in V5, with higher densities in autumn and spring when detritus is more abundant and temperature and humidity are favorable for decomposition processes. An increase in the intensity of mineralization and nitrification processes has been demonstrated with increasing temperature to about 30°C [46].

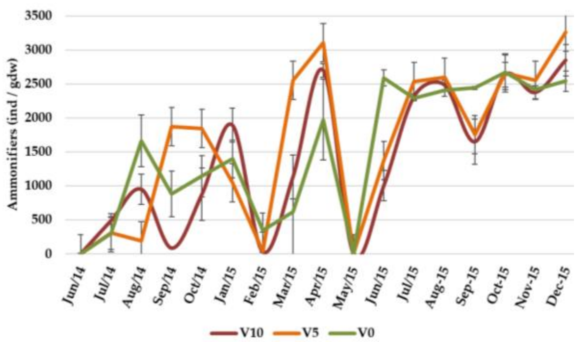

Figure 6. Ammonifying bacteria (individuals/g dry weight) from Vadu Lat site plots (June 2014 – December 2015).

Mineralization potential, which was determined in favorable conditions in the laboratory (high temperature and anoxic conditions), shows a positive correlation of mineralization rates with microbial population densities (Figure 7), with a better correlation for V5 plot.

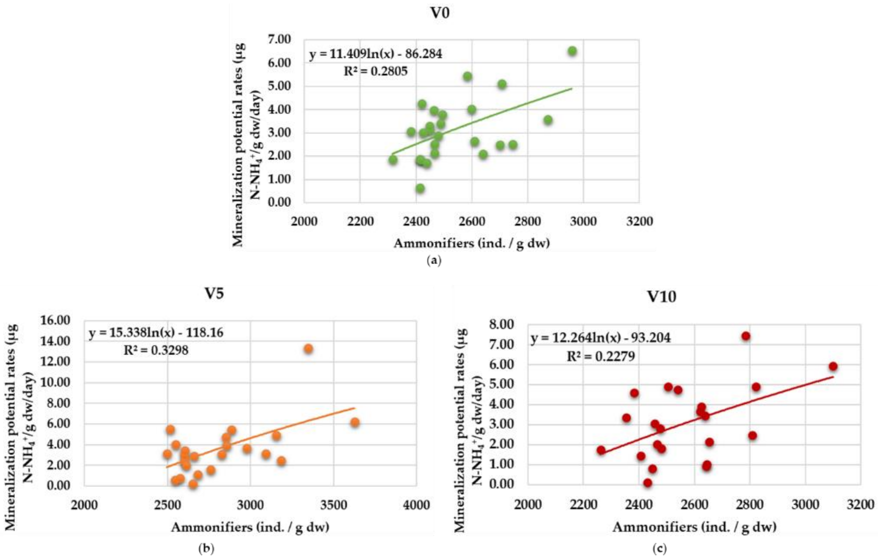

Figure 7. Mineralization potential rates (µg N-NH_4^+/g dry weight/day) vs ammonifiers soil density (individuals/g dry weight) for Vadu Lat fertilization plots (plot V0 - (a); plot V5 - (b); plot V10 - (**c**)) (June 2014 – December 2015).

The increase in population densities noticed in the second year of fertilization is a response to N addition. Nitrogen additions may alter microbial biomass and activity in several ways. The availability of N constrains primary production in most temperate ecosystems, and in turn, N may limit decomposition and microbial activity. Ågren et al. (2001) concluded that the major reasons for slower decomposition after N fertilization include increased decomposer efficiency, more rapid formation of recalcitrant organic matter, and decreased growth rate of decomposers [47]. In our case, adding inorganic nitrogen decreased the decomposition of organic matter in all plots, but the ammonifiers increased, so the efficiency decreased. We presume this can be the case of ammonium inhibition, the final product of the decomposition process [48]. These changes in decomposer growth rate and efficiency could be the consequence of shifts in microbial community structure and function and have important implications for C and N cycling at the ecosystem level [18].

Our results follow the statement in the literature that microbial activities are enhanced by increased soil organic content [49]. We obtained a positive correlation for V0 and V5 plots while plotting the ammonifier's population densities vs. soil organic matter (Figure 8). The correlation is negative and weaker in the case of V10, most probably due to a lower interval of available organic matter content.

According to previous studies, the intensification of agricultural practices through fertilization leads to increases in both nitrification and denitrification rates [50-52]. Studies by Van Breemen et al. (2002) showed about 37% reactive nitrogen losses through denitrification [40]. These values are confirmed by the study conducted by Seitzinger et al. (2006), which states that approximately 40% of reactive nitrogen from the soil is lost through the nitrate reduction process [53]. Immobilization of nitrates by microorganisms is a process that varies significantly across different types of terrestrial ecosystems, especially in the natural and semi-natural systems (undisturbed), but also in the agricultural soil ecosystems [54].

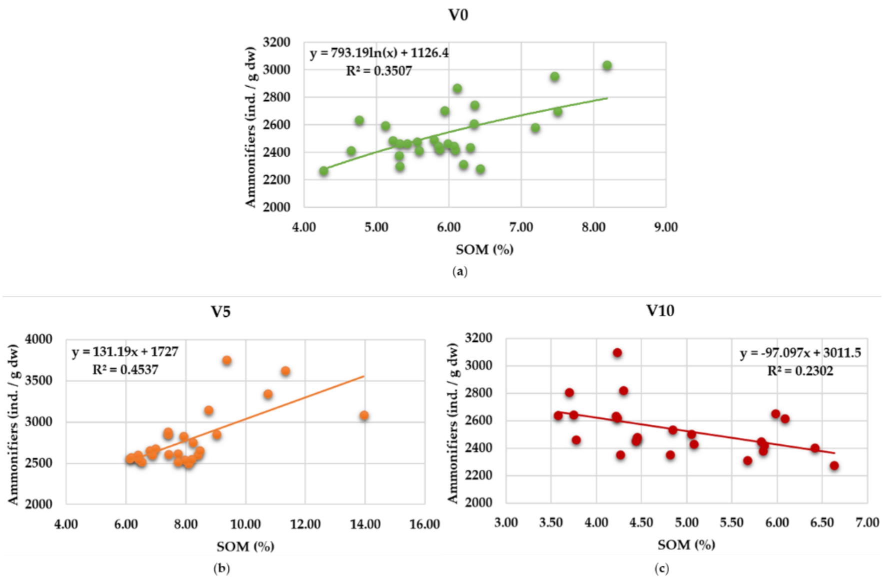

Figure 8. Ammonifiers soil density (individuals/g dry weight) vs soil organic matter (%) for Vadu Lat fertilization plots (plot V0 - (a); plot V5 - (b); plot V10 - (**c**)) (June 2014 – December 2015).

The assessment of the denitrifying community over the study period shows higher densities in the fertilized plots V5 and V10, which could be interpreted as a response to increased input of nitrate, the substrate of the denitrification process (Figure 9).

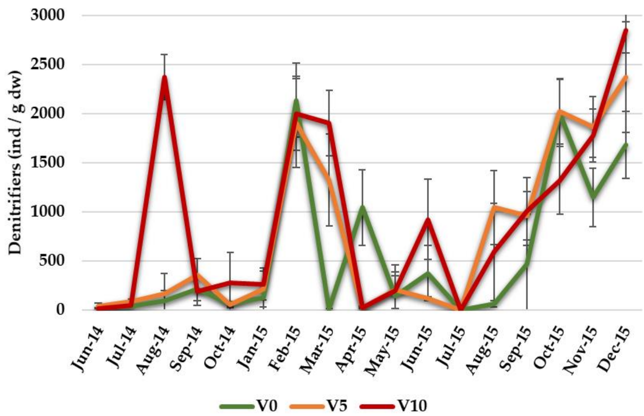

Figure 9. Denitrifying bacteria (individuals/g dry weight) from Vadu Lat site plots (June 2014 – December 2015).

However, the complex dynamic of denitrifiers is a consequence of various factors acting as driving forces, nitrate levels being less important for our site. This statement is supported by the negative correlation we obtained between denitrifiers and soil nitrate concentrations at low nitrate values (Figure 10). Despite the addition of equal amounts of nitrate and ammonium, nitrate stocks in soil are lower (<7 µgN-NH_4^+/g dry weight) as compared to ammonium stocks (10-20 µg N-NO_3^-/g dry weight) due to enhanced percolation of this very soluble chemical species. A decrease of microbial biomass with N fertilization was previously reported in the literature [34], and it remains unclear exactly why microbial biomass and soil respiration decreased under N fertilization [55,56], while microbially-mediated activities in soil were concomitantly enhanced. In particular, the roles of the key bacterial and fungal groups remain elusive in terrestrial ecosystems, and it is imperative to further study microbial functional group responses to N fertilization to elucidate their contributions to observed changes in microbial biomass.

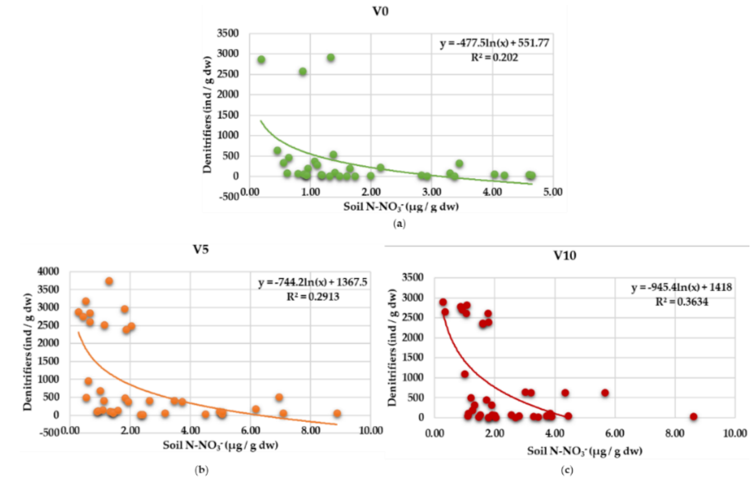

Figure 10. Denitrifiers soil density (individuals/g dry weight) vs soil N-NO_3^- (µg/g dry weight) for Vadu Lat fertilization plots (plot V0 - (a); plot V5 - (b); plot V10 - (c)) (June 2014 – December 2015).

Our study showed that SOM is the most important driving force for denitrifying community dynamics, proved by the positive correlation we obtained while plotting the denitrifiers' densities vs. organic matter, similar to ammonifiers dynamics (Figure 11).

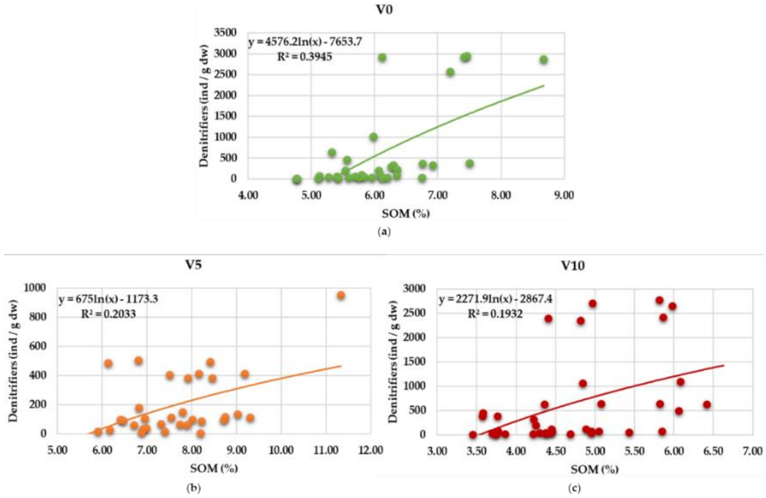

Figure 11. Denitrifiers soil density (individuals/g dry weight) vs soil organic matter (%) for Vadu Lat fertilization plots (plot V0 - (a); plot V5 - (b); plot V10 - (**c**)) (June 2014 – December 2015).

Some studies revealed that the influence of N amendments on bacterial diversity is inconsistent and site-dependent [57], based on finding that N fertilization reduces bacterial diversity [58,59]. In contrast, no changes in bacterial diversity among different fertilizer levels were observed [11]. Similar to our findings, soil organic matter was identified as one of the most predominant factors in explaining the differentiation of the microbial community across N fertilization intensities [57]. Literature reports support microbial communities' ability to cope with resource imbalances by regulating nitrogen use efficiency [60].

Microbial nitrogen use efficiency is important in understanding the nitrogen cycle. This parameter describes the partitioning of organic nitrogen between growth and the release of inorganic nitrogen to the environment.

4. Conclusions

The present study focused on the microorganisms involved in the terrestrial nitrogen biogeochemical cycle. The changes we observed during the study period are the first ecosystem responses; therefore, the magnitude of different monitored parameter variations was not too ample. A much more obvious response may be expected after a longer period.

Abiotic conditions are believed to be more important in shaping microbial communities rather than competitive interactions. Soil microorganisms are subjected to considerable seasonal fluctuations in environmental conditions such as temperature, water content, and nutrient availability.

The average of ammonifying and denitrifying bacteria presented shifts in community composition. These correlated with enhanced microbial activities and nutrient input from fertilization and litter decomposition.

The densities of microbial communities are influenced by fertilization, favoring denitrifiers and decreasing ammonifier densities.

The denitrifying bacteria were correlated with the concentration of nitrogen nitrate from the soil. In all three cases, the degree of correlation is higher in the systems fertilized with a higher concentration of ammonium nitrate.

Future studies should address microbial contribution in performing key processes of the biogeochemical cycle of elements. A better understanding of changes induced in soil properties and biogeochemical cycles by anthropogenic interventions through land-use changes and management strategies is required in order to explore the ecosystem's responses, their dynamics, and restoration management.

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Conflicts of Interest

The authors declare no conflict of interest.

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