

Associated microorganisms to the nitrogen cycle in soils under three systems of use: potato crop, livestock and páramo, in Los Nevados National Natural Park, Colombia

Microorganismos asociados al ciclo del nitrógeno en suelos bajo tres sistemas de uso: cultivo de papa, ganadería y páramo, en el Parque Los Nevados, Colombia

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Abstract

This study estimated the population density of microorganisms associated with the metabolism of nitrogen (N) in soils with three uses: potato crop, livestock and low intervention zones (high plains or páramo) in El Bosque district of Los Nevados National Natural Park in Colombia. The following microorganisms were indirectly quantified: amonifiers (AMO), proteolytic (PRO), ammonium oxidizing (BOA), nitrite oxidizing (BON) and denitrifiers (DEN) in soils of three farms located at different altitudes and under two climatic seasons. The non-parametric Kruskal-Wallis paired test with a significance level of $P \leq 0.05$ was used to detect statistically significant differences among abundances of functional groups of microorganisms. Then a principal component analysis (PCA) was performed to evaluate the relationships among different variables. The results showed differences among all the groups, with páramo soils having the lowest density of microorganisms in the soil. The PRO microorganisms were the most abundant in all soils, followed by DEN, with a higher density in the páramo ecosystem. The microbial population density was not significantly affected by altitude but showed some differences that did not have a unique behavior pattern. Additionally, differences were seen in the seasons, being higher in the dry season for the PRO, AMO, BOA and BON groups; and in the rainy season for DEN. This study provided knowledge of the relationships between land use and the presence of microorganisms associated with the nitrogen cycle.

Key words: Ammonium oxidizing, amonifiers, denitrifiers, nitrite oxidizing, proteolytic.

Resumen

En la vereda El Bosque del Parque Nacional Natural de Los Nevados (PNNN), Colombia, se estimó la densidad poblacional de microorganismos asociados con el metabolismo de nitrógeno (N) en suelos bajo tres sistemas de uso: cultivo de papa, ganadería y zonas poco intervenidas (páramo). Indirectamente se cuantificaron microorganismos amonificantes (AMO), proteolíticos (PRO), oxidantes del amonio (BOA), oxidantes del nitrito (BON) y denitrificantes (DEN) en suelos de tres fincas ubicadas a diferente altitud y en épocas seca y lluviosa. Se realizó la prueba no-paramétrica de Kruskal-Wallis por pares, con un nivel de significancia $P \leq 0.05$, para detectar diferencias estadísticamente significativas entre las diferentes abundancias de grupos funcionales de microorganismos. Luego, se realizó análisis de componentes

principales (ACP) para evaluar las relaciones entre las diferentes variables estudiadas. Los resultados mostraron diferencias para los grupos evaluados, siendo menor la densidad de microorganismos en suelos de páramo. Los PRO fueron más abundantes en todos los tipos de suelos, seguido de los DEN con mayor densidad en el ecosistema páramo. La densidad poblacional microbiana no fue afectada por la altitud, sin embargo se presentaron algunas diferencias que no mostraron un patrón único de comportamiento. Se observaron diferencias como resultado de la época climática, siendo mayor en la época seca para los grupos PRO, AMO, BOA y BON, y en época de lluvias para los DEN. Este estudio contribuye al conocimiento de las relaciones que existen entre el uso del suelo y la presencia de los microorganismos asociados con el ciclo de nitrógeno.

Palabras clave: Amonificantes, denitrificantes, oxidantes del amonio, oxidantes del nitrito, proteolíticos.

Introduction

The microbe community associated to the nitrogen (N) cycle represents an evolutive advantage by fixing atmospheric N₂ to convert it in forms assimilable for other organisms. The N is present in several forms, which are transformed during the cycle by the action of amonifiers (AMO), proteolytic (PRO), ammonium oxidizing (BOA), nitrite oxidizing (BON) and denitrifiers (DEN) microorganisms, among others (Loomis and Connor, 2002).

Knowledge on the diversity and metabolic capacity of the soil microorganisms associated to the N cycle is scarce. However, there are different methods to analyze the crop dependent and independent microbe communities (Nannipieri *et al.*, 2003). Among the first ones is the most-probable-number (MPN) technique which indirectly estimates the population density without doing a real counting of the microorganisms or colonies. Despite that the generated information is restricted only to communities that can be cultured and is sensitive to the inoculum density (Kirk *et al.*, 2004), this is a fast and cheap technique that generates useful information to estimate the abundance of the microbe community in the soil.

The type of soil is considered as a factor that directly or indirectly controls the structure of the microbe community in the soil (Han *et al.*, 2011). Microbe biomass is one of the indicators that better reflects responses to environmental changes, even better than physical and chemical parameters (Kaschuk *et al.*, 2011), and therefore, is fundamental for the evaluation of soil quality (Acosta-Martínez

et al., 2008).

Páramo is located between 3200 and 4500 MASL and constitutes a fragile ecosystem of great importance for water storage and hydric regulation (Hofstede *et al.*, 2003). In Colombia this ecosystem corresponds to an area of 30,000 km², being the country with the largest area of páramos (Ramírez, 2011) and in Los Nevados National Natural Park (PNNN) is the most representative ecosystem with an estimated area of 38,600 ha (66.21%) (PNN de Colombia, 2011).

In the Colombian Páramos potato is cultivated and livestock activities are developed, however, it is unknown the effect of these practices on soil properties and microbe communities. Therefore, the objective of this study was to estimate the population density of microorganisms associated to N metabolism in soils of the farm El Bosque in the PNNN, used for potato crop and livestock, compared to páramo soils with native vegetation. It is expected the establishment of possible relationships between soil use and presence of microorganisms that belong to different functional groups associated to N metabolism.

Materials and methods

Location and sample collection

Samples from rhizosferic soils for this study were taken in the farms Buenos Aires (3769 MASL), El Edén (3590 MASL) and La Secreta (3432 MASL) in the location El Bosque, Pereira, Risaralda. In each location were evaluated soil uses: páramo forest, potato (*Solanum tuberosum*) crop and livestock in the dry and

rainy season. Livestock is double-purpose (milk and meat production), with emphasis on milk production given the zone characteristics. Grasses sowed in the zone are Orchoro (*Dactylis glomerata*), Raygrass (*Lolium* sp.) and Plegadera (*Lachemilla* sp.). Páramo has predominantly vegetation of *Cortaderia selloana*, *Pernettya prostrata*, *Buddleja* sp., *Lupinus albus*, *Dendropanax* sp., *Chusquea* sp. in each one of the soil use types three samplings were performed, composed of 10 subsamples, for a total of 54 samples. In each sample was determined the abundance of five microorganism functional groups associated with N metabolism: PRO, AMO, BOA, BON and DEN.

Microorganism abundance and culture media

Microorganism abundance for the different functional groups was estimated by the MPN method (Cochran, 1950). Different culture media and carbon and N sources were used according to the functional group to study. For PRO and BOA the culture media was prepared using as basis the Winogradsky saline solution (Pochon, 1954) at a rate of 50 ml/l supplemented with a trace element solution (1 ml/l) (Balch *et al.*, 1977). For the PRO microorganisms it was used gelatin (30 g/l) as only carbon and N source for an incubation time of 15 days. For BOA microorganisms it was used calcium carbonate (1 g/l) and ammonium sulfate (0.5 g/l), as carbon and N sources respectively, and an incubation time of 30 days (Flórez-Zapata and Uribe-Vélez, 2011). For AMO microorganisms it was used as culture media and only carbon and N source bacteriological peptone (2 g/l) and an incubation time of 24 h (IGAC, 2006). To isolate BON it was used as culture media potassium nitrite (0.006 g/l), dipotassium phosphate (1 g/l), iron sulfate heptahydrate (0.03 g/l), sodium (0.3 g/l), magnesium sulfate heptahydrate (0.1 g/l), calcium carbonate (1 g/l) and calcium chloride (0.3 g/l); incubation time was 96 h (IGAc, 2006). Finally, for DEN microorganisms the culture media used was a mix of two solutions, A and B, with the following composition, solution A: potassium nitrate (2 g/l), asparagine (2 g/l), bromothymol blue 1% (10 g/l); solution B: sodium citrate (17 g/l), monopotassium phosphate (2 g/l), magnesium sulfate (2 g/l), calcium chloride hexahy-

drate (0.4 g/l), ferric chloride hexahydrate (0.1 g/l); incubation time was 72 h (IGAC, 2006) at 28 °C.

Microorganisms count

Presence or absence of organisms was determined using as characteristics the liquefaction of gelatin (PRO) and nitrite production, measured as Griess Ilosvay reactive (BOA) (Flórez-Zapata and Uribe-Vélez, 2011). The nitrite consumption was determined with the Griess Ilosvay reactive (BON), ammonium production by the Nessler reactive (AMO) and gas production using Durham bells and changes in blue coloration (DEN) (IGAC, 2006). Countings were expressed as colony forming units/g of dry soil (CFU/g).

Statistical analysis

Data analysis on the different abundances of the microorganisms functional groups was performed by Anova, however, due to the fact that the data do not support the normality principle, the non-parametric Kruskal-Wallis (KW) test by pairs with a significance level $P \leq 0.05$ was done, in order to detect statistical significant differences (Kruskal and Wallis, 1952). The above stated was done using the statistical software SAS 9.0 (009) and STATA 11.1(20059). Then, a principal component analysis (PCA) was performed to evaluate the relations between the different variables evaluated.

Results and discussion

Differences in the abundance of the different functional groups ($P = 0.0017$) were found (Table 1). In general, the microbe abundance was lower in páramo soil compared with potato crop and livestock, which did not show differences within them ($P = 0.289$). According to Roldán *et al.* (2009) this is possibly due to the fact that in páramo soils the densities of the functional groups associated with N cycle are lower because this use has high plant diversity, offering substrates that favor microorganisms' richness, but not diversity, which is different from the intensive management uses. However, the counting strategy by microorganisms culture could be a limiting factor for

Table 1. Density (CFU/g) of functional groups microorganisms associated with soil metabolism of N. the values are averages of replicates for: soil use (18), altitude (farm) (18) and season of the year (27).

Factors		Microorganisms density (CFU/g)				
		PRO	AMO	BOA	BON	DEN
Soil use	Páramo	35,059 a*	73 a	20 a	117 a	428 a
	Potato crop	213,052 b	213 b	1917 b	1297 b	1062 b
	Livestock	194,416 b	142 b	2001 b	741 b	2447 b
Altitude	3769 (Buenos Aires)	175,927 a	130 a	329 a	597 a	722 a
	3590 (El Edén)	142,235 a	107 a	923 ab	397 a	2032 b
	3432 (La secreta)	127,394 a	191 a	6959 b	891 a	1184 ab
Season	Rainy	77,392 a	76 a	-665 a	372 a	2020 a
	Dry	219,646 b	210 b	-395 a	1064 a	605 b

PRO:proteolitics, AMO: amonifiers, BOA: ammonium oxidizing bacteria, BON: nitrite oxidizing bacteria and DEN: denitrifiers. Values on the same column and factor followed by same letters do not significantly differ ($P \leq 0.05$)

possible recuperation and counting of those that are not cultured, which could be acting on a determined role within the N cycle in the soil, although they are not detected.

Páramo soils showed lower PRO densities compared with soils used for potato crop and livestock (Table 1) due, possibly, to the increment of nutrients that are easily available in these uses because of application of chemical synthesis fertilizers (N, P, K), as well as the mechanical labors that are done in these soils which increases their mineralization processes and, therefore, nutrient availability for microorganism growth. However, livestock soils show lower microorganism densities when compared to potato crop, as they are part of a rotation system in which potato is sowed for two years and then they are used for livestock (Ramírez, 2011).

PRO density did not change ($P > 0.05$) for the altitude effect, but for the effect of the season ($P = 0.0003$), being the abundance higher in the dry season (Table 1). Possibly, this is due to the fact that in the rainy season the water affects soil temperature, humidity and pH, factors that affect microbial activity (Roldán *et al.*, 2009).

AMO density changed for the soil use effect ($P = 0.001$) with lower density in the páramo ecosystem (Table 1). Calvo-Vélez *et al.*, (2008) state that in tropical soils the ammonification rate is affected by soil pH; thus, a pH between 5.5 and 6.0 is ideal for a maximum microorganism activity, but in more

acidic conditions the activity is reduced. In this study there were no differences found in pH due to soil use, therefore this factor might not be a limiting condition for ammonification in the páramo agroecosystem. One of the factors that can condition low AMO density in páramo is the low amount of substrate coming from PRO.

Differences in the AMO densities in the altitudes of this study were not significant ($P > 0.05$). On the other hand, in the dry season it was found a higher density than in the rainy season. This behavior of the AMO and PRO microorganisms is consistent as in soils with less PRO there were also less AMO, which is coherent because the PRO products are potential substrates for AMO. However, the later ones can be aerobic or anaerobic and in some cases they are able to form resistant reproductive structures that are viable during adverse periods when there is low humidity, low nutrient availability or flooding (Ponzuelo, 1991).

BOA concentrations were different ($P < 0.0001$) with respect to the soil use, being the livestock soils the ones with the highest densities (Table 1), but it was not the same in the potato crop ($P = 0.526$); similarly to the previous groups, BOA density was lower in the páramo ecosystem. Flórez-Zapata (2010) found 1.5×10^2 propagules/g of BOA in soil cultivated with potato, and Philippot and Germon (2005) consider that densities between 10^5 and 10^7 CFU bacterial/g are co-

common in agricultural soils

BOA densities in livestock soils could be associated with the increase in organic residues by cattle manure and animal urine, which are an ureic substrate that promotes microorganism growth (Orozco-Patio, 1999). The increase of microorganisms in cropping soils compared with páramo can be associated with fertilizers use, which increases the N availability that can be used as energy source by chemolithotrophs (BOA and BON) microorganisms (Roldán *et al.*, 2009). Additionally, the total biomass of the microorganisms involved to the N cycle increases with the intensity of soil use in grasses (Berner *et al.*, 2011). However, an amount of NH_4^+ larger than 800 mg/kg inhibits the development of autotrophic nitrifying microflora (Ponzuelo, 1991), which makes possible the use of the functional group (BOA) as indicator of changes in soil use (Roldán *et al.*, 2009).

BOA had an increase in population density as the altitude was reduced, this could be associated with the increase in temperature happening as altitude is reduced, approximately 0.57 °C each 100 m (Guevara-Díaz, 2003).

BON had low population density in páramo ecosystem (Table 1), which can be associated with low substrate coming for the oxidation of NH_4^+ that is done by BOA in this ecosystem.

Ponzuelo (1991) consider that the NO_2^- is a form easily unmovable due to its capacity to react with the soil organic matter (SOM), in special with phenolic compounds. These reactions happen in all type of soils and are favored by acidity and high SOM contents, as the ones found in páramo soils that range from 9.05 and 18.81% (data not shown).

BON neither show differences within altitudes of the farms in study nor with the period effect, however higher density was observed for the dry season (Table 1). DEN revealed lower density in páramo and higher in livestock ($P = 0.006$).

Denitrification is a heterotrophic process that occurs in anoxia or hypoxia conditions (Francis *et al.*, 2007) and is related to humid and high bulk density environments, as it is the case in the livestock soil in its first 20 cm

that are compacted, favoring high DEN densities (Ramírez, 2011). This also carries a reduction in soil microbe biomass (Li *et al.*, 2004) and an increase of the DEN microorganisms activity, which is close to 5% of the soil microbe biomass (Hai, 2009), that means, that both microorganisms are found in high densities in soils compared to AMO, BOA and BON. Flórez-Zapata (2010) considers that DEN respond to mineral fertilizer applications, because they obtain energy from these compounds. In this study, DEN were affected by the season of the year ($P = 0.001$), being its density higher in rainy seasons (Table 1). However, in páramo and livestock in the El Edén farm it was observed a higher activity in dry season, possibly due to low variation in soil humidity between seasons (data not published).

In the multivariate analysis were observed differences in microorganisms concentrations due to the soil use (Figure 1), where the páramo soil presents a different behavior compared to the potato and livestock soils; the later ones were correlated (0.8 and 0.73, respectively) to the axis 1, while in páramo the correlation to the axis 1 was negative (-0.48). a similar trend between the values of the livestock/potato crop systems could be due to the rotation between them that can cause fragmentation of the humus-allophanes complexes that favor SOM decomposition and increments in densities of microorganisms associated to these processes (Orozco-Patio, 1999).

AMO presented a higher density in soils used for livestock, this could be due to the fact that the cattle manner has compounds like methylamine which inhibits the AMO function (Orozco-Patio, 1999). In the same way happens in the crops because of the urea addition during fertilization, as it is expected, chemical fertilizers additions increase the nutrient availability (Roldán *et al.*, 2009). On the other hand, it was observed a lower density of microorganisms in the páramo ecosystem (Table 2 and Figure 1) which can be associated with the SOM stability, because humic substances predominate, allophane is present and do not allow decomposition of this matter due to their stable structure.

It was observed that PRO were more abundant in the páramo, which is important

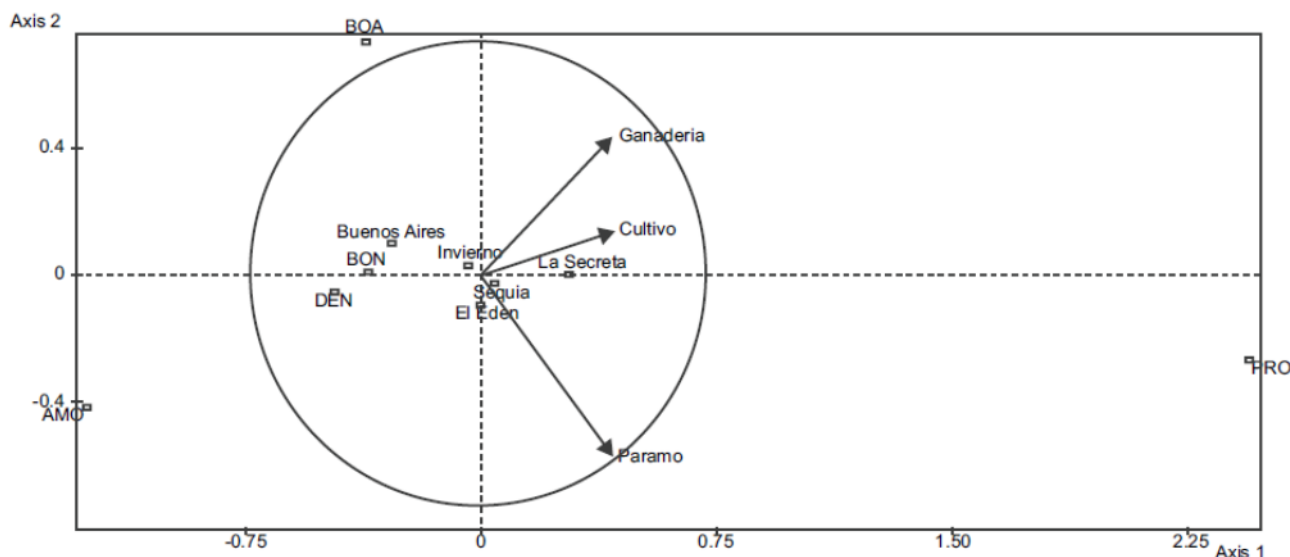


Figure 1. Principal component analysis for microorganism groups according to the soil uses evaluated. PRO:proteolitics, AMO: amonifiers, BOA: ammonium oxidizing bacteria, BON: nitrite oxidizing bacteria and DEN: denitrifiers.

because the proteins, which are the main N source in the natural ecosystems, are necessary for other processes in which the other functional groups are involved (Li *et al.*, 2004).

In the PCA done with the variables : PRO, AMO, BON, BOA and DEN (Table 3; Figure 2 and Figure 3) were taken the first three components because they explain 83.02% of the data variance. In the Figure 2 is observed that the more correlated variables in the axis 1 were PRO, AMO and BON, with values of 0.51; 0.74 and 0.76, respectively, while in the axis 2 are associated DEN and BOA with 0.86 and 0.37, respectively. In the PC1 is associated with mineralization processes, where the PRO fragment the organic matter in free amino acids, which later by the AMO activity are transformed in ammonium that is use as substrate for nitrification (Ponzuelo, 1991), a process that happens with the intervention of different microorganisms groups, ammonium

oxidizing bacteria (BOA) and nitrite oxidizing bacteria (BON) (Francis *et al.*, 2007).

Denitrification processes where DEN participates reducing NO_3^- to NO_2^- , N_2O and NO (PC2) are associated with BOA (Figure 2), it could be that under intensive agricultural conditions, increases on pH, temperature or soil porosity facilitate the conversion of ammonium to ammonia (NH_3^+) and this volatiles in a form of nitrogen limiting the substrate source for ammonia oxidation (Flórez-Zapata, 2010). It is possible that there is a relation between BOA and DEN, as proposed by Francis *et al.* (2007) and Le Roux *et al.* (2008) who consider that the ammonia oxidation process can happen under anaerobic conditions (Anammox), however, the role of this type of bacteria in the soil

Table 2. Variance and EIGEN values of the soil use of the principal component analysis.

Component	EIGEN Value	Accumulated variance (%)
1	2.27	75.69
2	0.37	88.19
3	0.35	100.0

Table 3. Variance and EIGEN values of the principal component analysis for the functional groups analyzed.

Component	EIGEN Value	Accumulated variance (%)
1	2.16	43.38
2	1.19	67.25
3	0.78	83.02
4	0.54	93.84
5	0.30	100.00

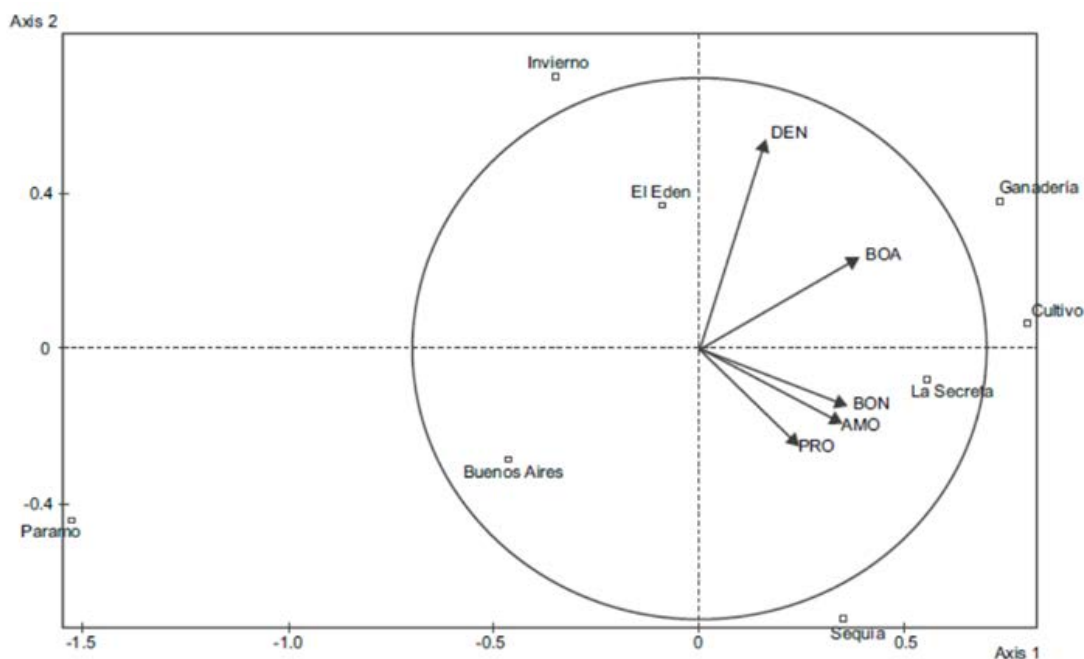


Figure 2. Principal component analysis for the functional groups evaluated. PRO:proteolitics, AMO: amonifiers, BOA: ammonium oxidizing bacteria, BON: nitrite oxidizing bacteria and DEN: denitrifiers. Axis 1 and 2. Ejes 1 y 2.

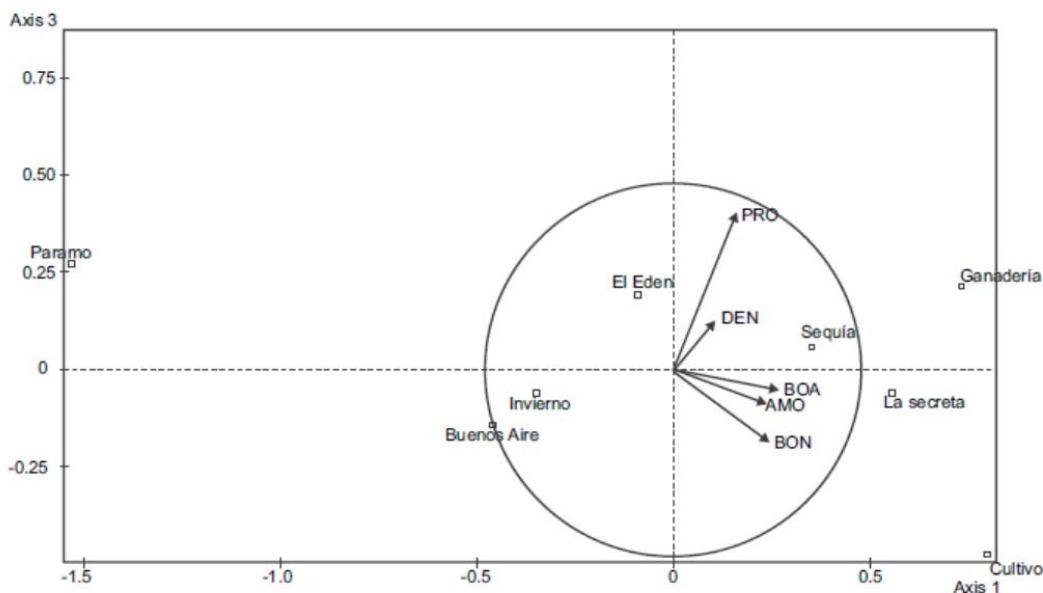


Figure 3. Principal component analysis for the functional groups evaluated. PRO:proteolitics, AMO: amonifiers, BOA: ammonium oxidizing bacteria, BON: nitrite oxidizing bacteria and DEN: denitrifiers. Axis 1 and 3.

nitrogen cycle is not clear yet.

In the Figure 3 is observed that PRO are correlated with the axis 3, compared with the DEN, thus, they apparently have an independent behavior. With the previous statement, is possible to relate each one of the

components with a process in the N cycle, where the axis 1 (PC1) represents ammonification and N oxidation processes, while the PC2 represents processes of N losses in the ecosystems by denitrification or Anammox, and the PC3 indicates processes related with protein fragmentation. In general, the PRO

microorganism populations show superior abundances in respect to the nitrifying and denitrifying microorganisms, and this is due to the fact that in the soils there is a high organic matter availability that favors the development of these microorganisms (Flórez-Zapata, 2010).

Conclusions

- There were differences between the population densities of the microbe communities in relation to the soil use and agricultural practices. The lowest density of functional groups was observed in the páramo ecosystem and the highest in potato and livestock uses, which could happen because of the use of fertilizers and mechanical management of the soil.
- PRO was the most abundant functional group in the evaluated ecosystems, indicating that in low intervene ecosystems those microorganisms mediate the process that gives energy to the other microorganisms of the N cycle.
- In general, microbe population density was not significantly affected by altitude; however there were small differences without any defined trend.
- The PRO, AMO, BOA, BON functional groups presented higher density in the dry season, which is associated with soil humidity, since these microorganisms are aerobic and its abundance is affected under hypoxia conditions in the soil. The opposite happened with DEN which are anaerobic microorganisms.
- The results showed a larger density of DEN in páramo ecosystem, compared to the other functional groups (except PRO), which suggests that in this ecosystem there is a negative balance in the N cycle, where the losses caused by denitrification can be larger than the earning by mineralization.

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