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Abundance of ammonia-oxidizing organisms across a gradient of preserved Brazilian Cerrado

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Abstract: The Brazilian cerrado comprises a diverse vegetation gradient with soils of different physicochemical properties. Previous studies have reported that these different physicochemical properties influence the responses of soil microbial properties. However, no study to date has evaluated the responses of ammonia-oxidizing organisms across the gradient of Brazilian cerrado. In this study, we measured the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) across the Cerrado gradient in northeast Brazil. Soil samples were collected in grassland, Cerrado sensu stricto and cerradao. The qPCR was performed using primers 341F/534R and Arch771F/957R for bacterial and archaeal 16S rRNA gene amplification, respectively. The archaeal and bacterial amoA gene amplifications were carried out using primers Arch-amoAF/AR and A189 and amoA-2R′, respectively. The abundance of archaea, AOA, AOB, and AOA/AOB ratio varied according to the sites; while the abundance of bacteria that did not vary between sites. Usually, AOA and AOB were highest in cerradao than grassland. There were significant correlations between physicochemical and microbial properties and the multivariate analysis clearly separated the sites according to physicochemical and microbial properties. Interestingly, all sites were also clearly separated between the dry and rainy seasons, with soil moisture appearing to be one of the dominant factors influencing cluster separation. In conclusion, the different physicochemical properties of the soil found across the gradient influenced the ammonia-oxidizing archaea, while ammonia-oxidizing bacteria was not driven by these properties.

Key words: Ammonia-oxidizing archaea, ammonia-oxidizing bacteria, biodiversity, soil microorganisms.

Introduction

The Brazilian cerrado comprises a diverse plant gradient from 'campo graminoide' (grassland formation), through typical 'cerrado sensu stricto' (savanna formation with trees and shrubs up to 10 m high and with grass), to 'cerradao' (forest formation with trees up to a height of 20 m) (Coutinho 1978). Previous studies have shown that the soils under these diverse formations of cerrado display different physicochemical properties (Lucena *et al*. 2014; Ruggiero *et al*. 2002), which may have strong influence on soil microorganism (Philippot *et al*. 2013).

Soil microorganisms are thought to constitute the largest reservoir of soil biodiversity in natural ecosystems (De Mandal *et al*. 2015) and are involved in vital ecological processes (Rodrigues *et al.* 2013).

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Table 1. Average of soil physicochemical properties at different sites across the gradient of cerrado.

	Grassland	Cerrado SS	Cerradao	Grassland	Cerrado SS	Cerradao Cerado
	Rainy			Dry		
Temperature (°C)	27[a]	29[a]	29[a]	33[a]	30[a]	31[a]
Moisture (%)	7.8[b]	10.1[a]	11.2[a]	0.35[b]	0.58[a]	0.73[a]
NT (g kg^{-1})	0.15[b]	0.29[a]	0.34[a]	0.20[b]	0.37[a]	0.40[a]
TOC (g kg^{-1})	4.1[b]	6.8[a]	7.7[a]	4.3[b]	8.3[a]	9.5[a]
pH	4.8[a]	4.7[a]	4.7[a]	4.8[a]	4.6[a]	4.7[a]
P (mg kg^{-1})	3.4[b]	4.4[a]	4.6[a]	2.3[b]	3.2[a]	3.3[a]
Na ($cmol_c$ kg^{-1})	0.46[a]	0.44[a]	0.35[b]	0.64[a]	0.15[b]	0.16[b]
Ca+Mg ($cmol_c$ kg^{-1})	0.11[b]	0.36[a]	0.25[a]	0.23[b]	0.67[a]	0.41[a]

The oxidation of ammonium to nitrite, the rate-limiting step of nitrification (Carney *et al.* 2004), is an important ecological process carried out by ammonia-oxidizing bacteria (AOB) and archaea (AOA) in various soil environments (He *et al.* 2012; Leininger *et al.* 2006). Previous studies have evaluated the abundance of ammonia-oxidizing bacteria and archaea in several different ecosystems (Boyle-Yarwood *et al.* 2008; Li *et al.* 2011), and demonstrated that AOA and AOB are regulated by soil physiochemical properties, such as moisture, organic matter, salinity, and soil pH (Bernhard *et al.* 2010; Nicol *et al.* 2008; Tourna *et al.* 2011).

In the Brazilian Cerrado, studies of soil microbial properties have focused on responses of soil microbial biomass and activity (Nardoto & Bustamante 2003; Mendes *et al.* 2012), and microbial community structure (Araujo *et al.* 2012; Castro *et al.* 2016) to the different vegetation formations. These studies have reported that different physicochemical properties found in soils from different vegetation of Cerrado influenced the responses of soil microbial properties. However, it was unclear how specific functional groups, such as the ammonia-oxidizing organisms, would behave across the gradient of native Brazilian Cerrado.

Materials & Methods

The study was conducted within Sete Cidades National Park (PNSC) (04°02'–08'S and 41°40'–45'W), located in the northeastern state of Piauí. The park covers an area of 6,221 ha. There are two distinct seasons (wet and dry) during the year, with annual average temperatures of 25 °C. The area has an annual average rainfall of 1,558 mm distributed in February, March and April.

Within the Cerrado we evaluated preserved sites (each 1,000 m^2) that belong to a Brazilian government long-term ecological program (PELD-CNPq), across a gradient of different cerrado formations ranging from grassland, cerrado sensu stricto and cerradao. In brief, grassland is covered by a continuous grass stratum which does not exist in Cerradao; while Cerradao is covered by woody stratum with varying density of shrubs and trees which is absent in Grassland. Intermediary, Cerrado sensu stricto is covered by grass, shrubs, low trees and woody stratum.

Each site was divided in three transects (for replication) where soil samples were collected at 0–20 cm depth (three points per transect which were mixed to obtain a composite sample per transect) in March (wet season) and September (dry season) in 2014. All soil samples were immediately stored in sealed plastic bags and transported in an ice box to the laboratory. A portion of the soil samples was stored in bags and kept at −20 °C for DNA analysis and another portion was air-dried, sieved through a 2 mm screen and homogenized for chemical analyses.

Soil chemical properties were determined and measured using standard laboratory protocols. Soil pH was determined in a 1:2.5 soil/water extract. Available P and exchangeable K^+ were extracted using Mehlich-1 extraction method and determined by colorimetry and photometry, respectively (Tedesco *et al.* 1995) (Table 2). Total organic C (TOC) was determined by the wet combustion method using a mixture of potassium dichromate and sulfuric acid under heating (Yeomans & Bremmer 1998).

Soil DNA was extracted from 0.5 g (total humid weight) of soil using the Power Soil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The DNA extraction was performed in triplicate for each soil sample. The quality and relative quantity of the

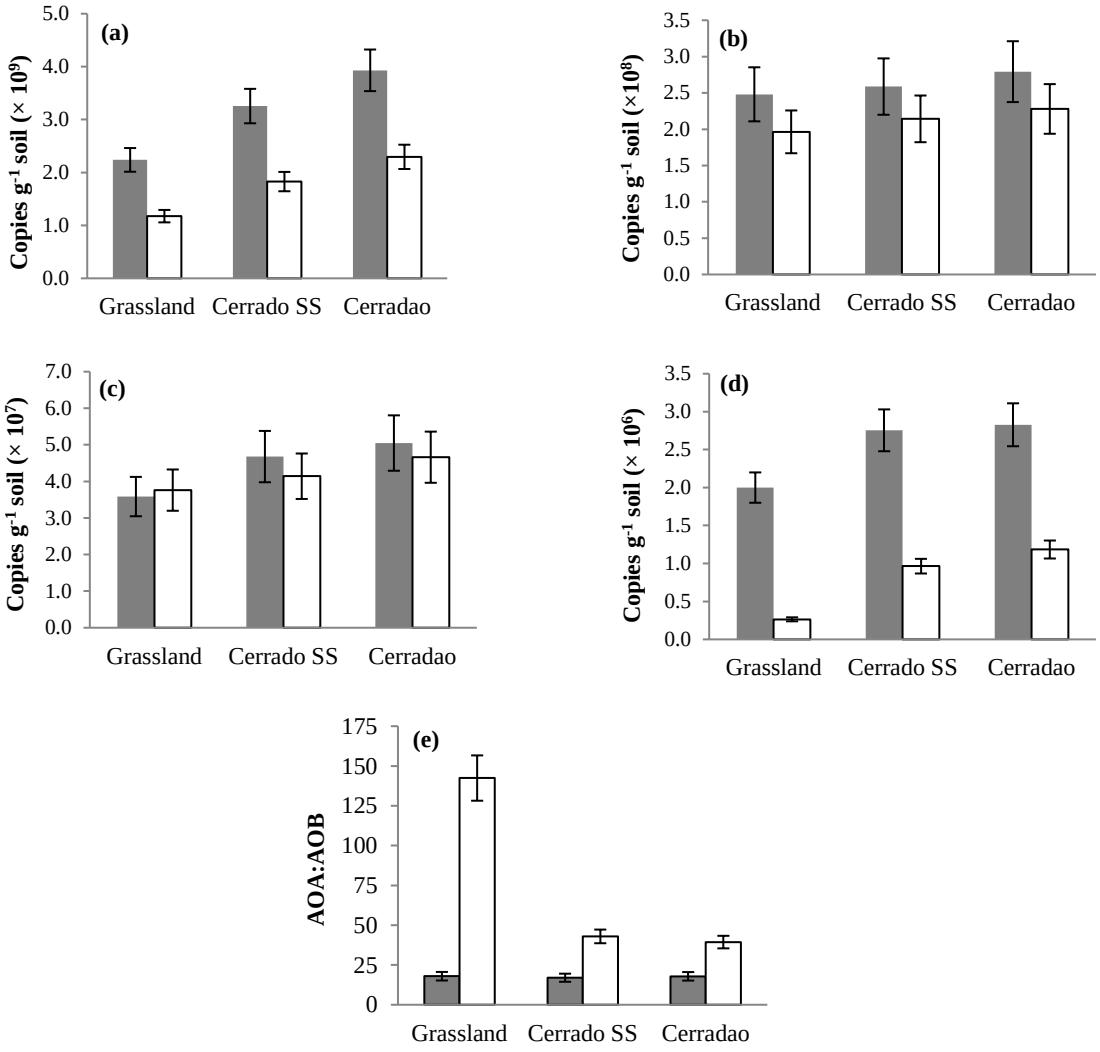

Fig. 1. Abundance of bacterial 16S rRNA (a) and archaea 16S rRNA (b), gene copy number for AOA (c) and AOB (d), and the ratio of AOA/AOB (e) across a gradient of cerrado in the rainy season (filled bars) and in the dry season (open bars). Error bars indicate one standard deviation.

extracted DNA was determined using a Thermo Scientific NanoDrop 2000.

The qPCR was performed on an Applied Biosystems (Applied Biosystems, NJ, USA) ABI 7300 sequence detection system using SYBR green detection. The qPCR was performed in 20 µL reaction mixtures containing the following components: 10 µL of SYBR GreenER™ qPCR SuperMix (Invitrogen, NJ, USA), and 0.5 µM of each primer. Primer set 341F/534R was used for bacterial 16S rRNA gene amplification (Lopez-Gutierrez *et al.* 2004; Muyzer *et al.* 1995). The qPCR assay to estimate archaeal 16S rRNA gene content used the primer set Arch771F/957R (Ochsenreiter *et al.* 2003). The archaeal amoA gene amplifications were carried out using primers Arch-amoAF/AR (Francis *et al.* 2005), while the primers A189 and amoA-2R′ were used for the bacterial amoA gene quantification (Holmes *et al.* 1995; Okano *et al.* 2004).

A melting curve analysis was performed after each assay to ensure that only the products of the desired melting temperature were generated from the SYBR green qPCR. The R^2 values for the standard curves were 0.99 or better for all runs. All reactions were run in triplicate with a standard curve spanning 10^1–10^6 copy numbers for bacterial and archaeal 16S rRNA genes, or 10^0–10^5 copy numbers for bacterial (AOB) and archaeal *amoA* (AOA) genes. The standard curves for quantifying

gene copy numbers were determined by cloning the PCR products in a plasmid using the procedures reported by Okano *et al.* (2004). The population sizes of total bacteria, archaea, AOA and AOB were estimated as the normalized copies per gram of dry soil.

The results are expressed on the basis of oven-dry soil and all measurements were performed for three replicates per site. Split plot analysis of variance (ANOVA) was used to test the effect of different sites (grassland, cerrado SS and cerradao), season (dry and rainy season) and the interaction between sites and season on the evaluated soil microbial properties. A non-metric multi-dimensional scaling (NMS) ordination, with Sorensen distances, was performed to ordinate the sites according to the physicochemical and microbial properties of the soil. First, the data were normalized and one secondary matrix was used to correlate the physicochemical and microbial properties. The significance of correlations was estimated through of VassarStats web-page (http://vassar.net/rsig.html). Statistical differences were estimated by using the multi-response permutation procedure (MRPP), which showed the differences ($P < 0.05$) between sites in the NMS analysis. The P values were adjusted by Bonferroni correction and all analysis were performed using the PC-ORD v.6.0 program.

Results

The physicochemical properties varied across the gradient of cerrado (Table 1), showing that cerrado SS and cerradao presented similarities and were different from the grassland. The abundance of archaea, AOA, AOB, and AOA/AOB ratio varied according to location (Fig. 1). The exception was the abundance of bacteria that did not vary between sites (Fig. 1a) or seasons. In the rainy season, the archaea abundance did not vary between sites, while in the dry season, the highest value was found in grassland (Fig. 1b). AOA gene copies were highest in cerradao and lowest in grassland during the rainy season, while the values were highest in grassland in the dry season (Fig. 1c). AOB gene copies did not vary between sites, in the rainy season, while in the dry season, the highest values were found in cerradao (Fig. 1d). Interestingly, the AOA/AOB ratio was highest in the cerradao in the rainy season; however, in the dry season, the highest values were observed in grassland (Fig. 1e).

NMS analysis explained 82% of the total variation by the first two axes (Fig. 2; Table 2). The first axis explained 42% of the variation and was positively correlated with Ca+Mg, TOC, TN, N:P, and C:P; and negatively correlated with Na, AOA/AOB ratio, and the abundance of archaea. The second axis explained 40% of the variation and was positively correlated with AOB, AOA, soil moisture, and P; and negatively correlated with N:P, and C:P. Samples from within each individual vegetation region clustered together, and differentiated the sites along the first axis in the dry season, and along both axes in the rainy season (Fig. 2). All sites were also clearly separated between the dry and rainy seasons, with soil moisture appearing to be one of the dominant factors influencing cluster separation (Fig. 2).

Table 2. Pearson correlation coefficients (r) between microbial and chemical properties of the soil, and axes 1 and 2 of ordination NMS.

Variables	Axis 1	Axis 2
16S Bac	0.46^{ns}	−0.05^{ns}
16S Arch	−0.61**	0.38^{ns}
AOA	−0.42^{ns}	0.65**
AOB	0.16^{ns}	0.76***
AOA/AOB	−0.61**	−0.21^{ns}
pH	−0.33^{ns}	−0.06^{ns}
Al	0.46^{ns}	−0.16^{ns}
Ca + Mg	0.93***	−0.27^{ns}
K	0.20^{ns}	0.14^{ns}
P	0.06^{ns}	0.87***
TOC	0.74***	0.02^{ns}
TN	0.71***	0.04^{ns}
C:N	−0.15^{ns}	0.03^{ns}
N:P	0.63**	−0.53*
C:P	0.71***	−0.58*
Moisture	−0.43^{ns}	−0.74***

*,**,***, represent significance at P<0.05, 0.01 and 0.001, respectively and, ns- non-significant. 16S Bac-abundance of bacteria; 16S Arch-abundance of archaea; AOA-ammonia-oxidizing archaea; AOB-ammonia-oxidizing bacteria; Al-aluminum; K-potassium; P-phosphorus; TOC-total organic C; TN -total N.

Discussion

The ammonia-oxidizing organisms presented different behaviors, according to with differences in soil physicochemical properties across the gradient of Brazilian cerrado. Interestingly, the abundance of archaea was influenced by the physicochemical properties found at the different sites, while that

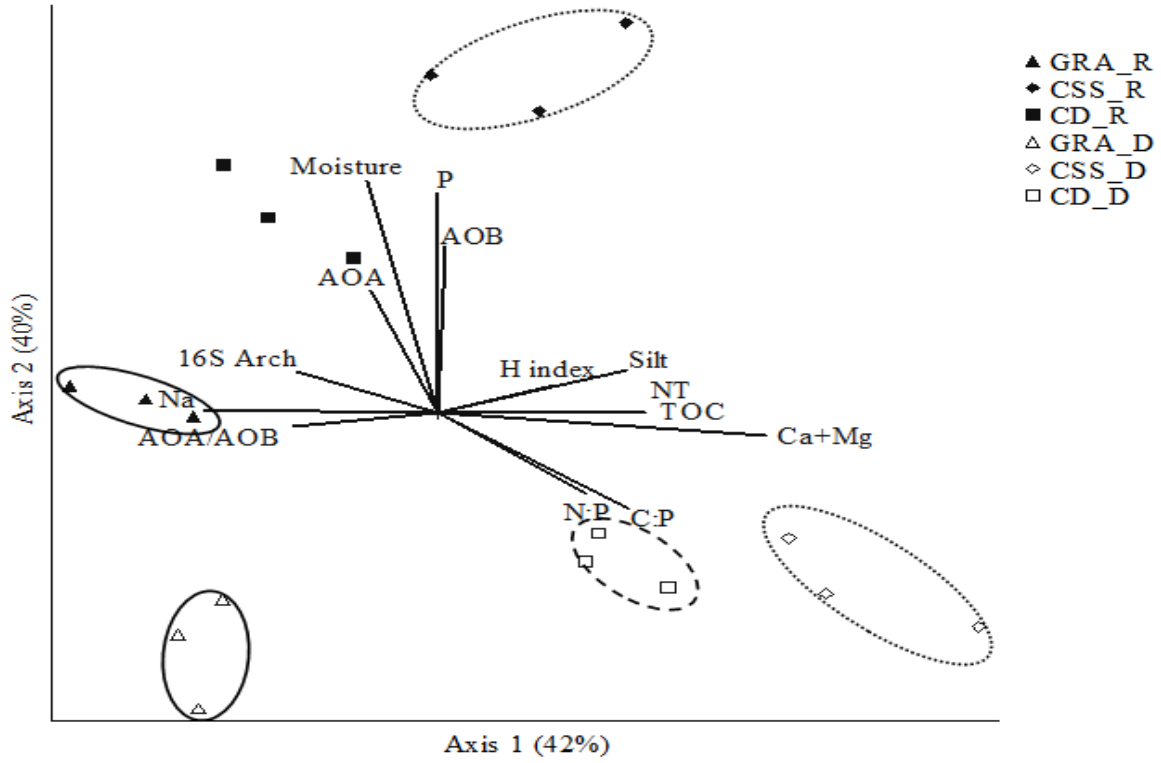

Fig. 2. NMS ordination of soil samples across a gradient of cerrado according to ammonia-oxidizing organisms and plant-soil properties. GRA_R-Grassland (rainy season); CSS_R-Cerrado SS (rainy season); CD_R-Cerradao (rainy season); GRA_D- Grassland (dry season); CSS_D – Cerrado SS (dry season); CD_D-Cerradao (dry season); 16S Arch- abundance of archaea; AOA-ammonia-oxidizing archaea; AOB-ammonia-oxidizing bacteria; Al-aluminum; K-potassium; P-phosphorus; TOC- total organic C; TN -total N.

the abundance of bacteria was not driven by these physicochemical properties. As shown in Table 1, the sites presented different physicochemical properties, such as moisture, P, Na, C:N, and N:P, and these characteristics, associated with acidic soils, may explain the different responses of archaea than bacteria (Shen *et al.* 2012). He *et al.* (2012) reported that acidic conditions contributed for a shifting in AOA community but not in AOB in soil with different levels of nutrients. In contrast, Shen *et al.* (2008) observed that AOB were influenced, and not AOA, in alkaline soils with different levels of nutrients. It means that AOA are more active and responsive than AOB in acidic soils similar with our conditions. Also, our results showed that in the dry season, archaea had the highest abundance in grassland, confirming an important characteristic of archaea to adapt and grow under unfavorable environmental condition (Lamb *et al.* 2011; Tourna *et al.* 2011) as found in grassland during the dry season, i.e. low soil moisture.

AOA gene copies presented highest values in cerradao and grassland, in the rainy and dry season, respectively, and these results suggest that AOA were influenced by both highest and lowest soil moisture. In contrast, AOB were positively influenced by the highest soil moisture found in cerradao. On one hand, our results indicate that soil moisture is an important driver of AOA and AOB abundance in soils from Brazilian cerrado. On the other hand, we found that AOA and AOB responded differently to soil moisture. These results are consistent with previous studies in other environments that observed that AOA and AOB respond differently to changes in soil moisture (Auyeung *et al.* 2015; Bustamante *et al.* 2012). In particular, AOB were more sensitive to soil moisture changes than AOA, and this behavior suggests greater versatility under limiting environmental conditions in AOA than AOB.

The pattern of AOA distribution contributed to the highest values of AOA/AOB ratio found in cerradao and grassland, in the rainy and dry

season, respectively. Finally, AOA were more abundant than AOB in all sites, confirming the predominance of AOA over AOB in several soils (Chen *et al.* 2013; Leininger *et al.* 2006; Shen *et al.* 2008).

The physicochemical properties of the soil displayed varying influence on the abundance of ammonia-oxidizing organisms. NMS analysis showed different physicochemical variables influencing the abundance of archaea, AOA, and AOB gene copies. The first, which explained 42% of variation, showed some chemical properties, related with soil organic matter, positively clustered with cerrado SS and cerradao and negatively correlated with the abundance of archaea, and AOA/AOB, which were clustered with grassland (Fig. 2). These results indicate that cerrado SS and cerradao, with similarity in some soil chemical properties, showed highest levels of soil organic matter than grassland, and contributed to the negative correlation with archaea, confirming that organic matter-poor conditions found in Grassland favored archaea than bacteria. Similar findings were also reported by Banning *et al.* (2015) who found negative correlations between soil organic matter content and the abundance of archaea and AOA. Interestingly, we observed that the nutrient stoichiometry (i.e. N:P, and C:P ratios), which were higher in cerrado SS and cerradao, correlated negatively with the abundance of archaea suggesting that these microbes are not driven by these properties. In fact, nutrient stoichiometry and the quality of substrate (oligotrophy or eutrophy) influence ammonia-oxidizers organisms (Bollman *et al.* 2014). Usually, AOB are influenced by nutrient enrichment and present higher abundance in soil with higher N and P content, i.e. eutrophic soils (Bollman *et al.* 2014). Also, Lage *et al.* (2010) have suggested that a fine-scale genetic differences with the AOB than AOA regulate their higher ability to use N and P.

On the other hand, Na was an important chemical variable correlated with archaea, and AOA/AOB ratio, and these results agree with previous studies which also found positive correlations between salinity and the abundance of ammonia-oxidizing archaea (Bernhard *et al.* 2010; Caffrey *et al.* 2007). Bernhard *et al.* (2010) reported that the abundance of archaea was higher than that of bacteria along a salinity gradient, and that increased salt concentrations increased the AOA/AOB ratio. The higher abundance of AOA in the presence of salinity may be explained by their different proteins that have a number of adaptations and allow them to stabilize their biomass under high concentrations of inorganic salts (Reed *et al.* 2013).

The second axis, which explained 40% of the variation, indicated that AOB, and AOA were influenced by soil moisture, and P content. Also, AOA, and AOB were clustered with cerrado SS and cerradao which presented the higher values of soil moisture and P than grassland. The results suggest that P is an important chemical variable influencing AOA and AOB in soils from Brazilian cerrado. Although few studies have been done investigating the effect of P on ammonia-oxidizing organisms (Dodor & Duah-Yentumi 1999; Peng *et al.* 2012), these reports showed P as a positive variable influencing the ammonia-oxidizing organisms. Specifically, P favored the growth of AOB since the availability of nutrients, such as P, drive the bacteria communities that are nutrient limited (DeBruyn *et al.* 2004). Soil moisture was another important factor contributing for differences in the AOA and AOB, as soil moisture is an important driver of soil microbial community (Tabuchi *et al*. 2008). Reasons for the positive effect of moisture on AOA and AOB in cerrado SS and cerradao, which presented the highest organic matter content, may include: (a) soil moisture increased the availability of organic matter from woody debris and stimulated the abundance of AOA and AOB (Eaton & Chassot 2012) and (b) soil moisture accelerated soil mineralization rates and increased ammonia availability, thus increasing AOB abundance (Chen *et al.* 2014).

In the dry season, the sites were clustered along the first axis with Grassland separated from cerrados SS and cerradao (Fig. 2). In the rainy season, the sites presented similar pattern to those observed for the dry season; however, all sites were distributed along both axes. The results showed that the sites were clearly separated according to physicochemical and microbial properties with the formation of six different clusters. In the dry season, the grassland was strongly separated from the others sites (cerrado SS and cerradao), which were clustered somewhat more closely. However, in the rainy season, all sites were uniformly separated. Thus, these results confirm previous findings that soil moisture, which is influenced by the seasons, is an important factor for the responses of soil microbial properties in Brazilian cerrado (Nardoto & Bustamante 2003; Mendes *et al.* 2012).

Studies regarding the effect of environmental factors on ammonia-oxidizers organisms have shown that AOA and AOB occupy different niches

differentiation (Beman *et al.* 2008; Erguder *et al.* 2009; Francis *et al.* 2007). AOA inhabit a significant range of environmental conditions (Erguder *et al.* 2009) and outcompete AOB in nutrient-poor (Beman *et al.* 2008) and acidic (Zhang *et al.* 2012) environments. On the other hand, AOB are frequently found in environments with higher substrate availability (Beman *et al.* 2008).

Conclusion

We conclude that soil physicochemical properties influence ammonia-oxidizing organisms across the gradient of Brazilian cerrado. However, the different physicochemical properties of the soil found across the gradient influenced the ammonia-oxidizing archaea, while ammonia-oxidizing bacteria were not driven by these properties. These findings highlight the separation of ammonia-oxidizing bacteria and archaea status in this gradient of Brazilian cerrado.

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