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Resumo Geral

A interação entre as variáveis do solo e a microbiota influencia os processos que ocorrem no solo, tanto que, em ambientes terrestres o N é reciclado primariamente pela microbiota. No ciclo do N, a nitrificação é a etapa em que nitrato se torna disponível no solo para as plantas, mas também N é perdido por lixiviação de nitrato ou pela emissão de gases nitrogenados. Entretanto, as mudanças climáticas, a modificação do uso da terra e a aplicação de fertilizantes nitrogenados veem alterando a dinâmica de N. Um especial interesse é direcionado à maior savana na América do Sul, o bioma tropical sazonal seco que é o Cerrado, cuja paisagem vem sendo alterada pela agricultura. Fazendo uso da técnica de metagenômica, os atributos funcionais da microbiota do solo do Cerrado quanto ao ciclo do N foram comparados entre dois parques de conservação do bioma, distantes 500 km entre si, com variação na textura e no conteúdo de água do solo. Os tipos de vegetação amostradas dentro de cada parque mascararam os efeitos de altitude e distância entre os parques, e todas as amostras apresentaram uma maior abundância de genes para assimilação de amônia e amonificação. Isso corrobora a literatura encontrada sobre o metabolismo de amônia como forma principal de N no Cerrado. Em particular, o Campo limpo alagado, presente somente em um dos parques, apresentou a maior abundância de genes fixadores de nitrogênio. Ainda, foram detectados genes para denitrificação, mas somente dois hits foram observados para nitrificação. Sucessivamente, foi acessado o impacto do manejo do solo sobre a abundância de *Archaea* e *Bacteria* oxidantes de amônia por quantificação do gene marcador *amoA* ao longo do cultivo da soja no bioma Cerrado. A análise molecular, tal como as técnicas clássicas e de isótopos mostraram um maior conteúdo de C orgânico e de NH_4^+-N no pousio em comparação à área nativa de reserva legal adjacente ao plantio da soja. De mesma forma, observou-se um aumento na abundância de oxidantes de amônia e da taxa de nitrificação no solo agrícola em comparação à área nativa, com a menor razão amônia/nitrato observada no solo após revolvimento. A abundância de AOB apresentou correlação com o aumento de pH ao longo do cultivo da soja. Experimentos seguintes testaram o efeito de água e de pH em microcosmos contendo solo do Cerrado, tal como a possível inibição de nitrificação em *slurries* contendo uma mistura de solo do Cerrado com um solo agrícola (Craibstone) com reconhecida atividade de oxidação de amônia. No entanto, o acúmulo de NO_3^- estava abaixo do nível de detecção na maior parte das amostras, tanto naquelas com aumento no teor gravimétrico de água ou com aumento de pH, independente da alta concentração de amônia. A nitrificação não foi inibida nas misturas de *slurries* incubadas, e, ainda, após 21

dias de incubação foi possível detectar transcritos de *amoA* de AOA no *slurry* de solo de Cerrado. Os perfis de DGGE mostraram um maior número de bandas de AOA *amoA* nos *slurries* de Craibstone e das misturas dos dois solos, do que o perfil observado nos *slurries* incubados somente com solo do Cerrado. Considerando o exposto acima, este foi o primeiro trabalho apresentado sobre o metabolismo de N e mais especificamente sobre a oxidação de amônia, utilizando dados de metagenomas e de PCR em tempo real. A baixa detecção de nitrato nas amostras de campo e de incubações em laboratório sugerem que algum outro mecanismo ocorre nos solos do bioma Cerrado no sentido de preservação de N inorgânico preferencialmente na forma de amônia. Sugerimos que a nitrificação depende da presença de oxidantes de amônia, mas também da composição da comunidade microbiana, sendo que a sua diversidade afeta a dinâmica de N no solo. Provavelmente condições abióticas e bióticas influenciam na limitação de crescimento da comunidade de oxidantes de amônia autotróficos no Cerrado. Por exemplo a competição por amônia entre esses oxidantes autotróficos e plantas ou com microorganismos heterotróficos. Ainda a redução dissimilatória de nitrato a amônia ou a imobilização abiótica de nitrato podem influenciar o desenvolvimento daquela comunidade

General abstract

Interactions between soil characteristics and microbiota influence the processes in soil ecosystem, as the terrestrial N is primarily cycled by the microbiota. In the N cycle, nitrification enables plants' access to nitrate, although N can be lost through nitrate leaching, or N trace gas emission. These N dynamics are being disturbed by climate change, land use modification and the employment of nitrogenous fertilizers. A special interest goes to the largest savanna in South America, the seasonally dry Cerrado biome, where agriculture is changing the biome landscape. Shotgun metagenomics was used to compare the functional attributes of N cycling from the soil microbiota present in two conservation parks of the Cerrado biome, 500 km distant from each other, with varying soil texture and water content. Types of vegetation sampled within each park masked the altitude and distance effects, but all samples showed higher abundance of genes for assimilation of ammonia and ammonification. This corroborates Cerrado literature of ammonia as the main soil N form. In addition, a flooded grassland presented the highest abundance of N fixation genes. Despite the detection of denitrification genes, only two hits for the nitrification process were described. Subsequently, we assessed the impact of soil management on the abundance of *Archaea* (AOA) and *Bacteria* (AOB) ammonia oxidizers by quantification of the marker gene (*amoA*) during different stages of soybean cultivation within the Cerrado. Molecular analysis and classic and isotope techniques exhibited higher content of organic C and NH_4^+-N during fallow than in the adjacent undisturbed field, and an increase in ammonia oxidizers abundance and nitrification rates in the agricultural soil than in the undisturbed site, with the lowest ammonium/nitrate ratio in tilled soil. AOB abundance was correlated with the increase in pH during soybean cultivation. Further experiments tested the effect of moisture and pH in microcosms containing Cerrado soil, and the possible nitrification inhibition in slurries assembled with a mixture of Cerrado and agricultural soil known for actively oxidizing ammonia (Craibstone soil). Nevertheless, very little NO_3^- accumulation was observed in Cerrado microcosms with either increasing moisture or pH, despite high ammonia concentration. Nitrification was not inhibited in the mixed soil slurries, and after 21 days it was possible to detect the activity of AOA with the quantification of *amoA* transcripts. Moreover, DGGE profiles showed a higher number of AOA *amoA* gene in the Craibstone-only slurries and similar to the mixed slurries, but lower in the Cerrado-only slurries. This was the first assessment of the N metabolism with metagenomic data and qPCR for ammonia oxidation in the Cerrado. However, the little accumulation of NO_3^- in the field soils or in the treated microcosms or slurries advocates that some other mechanism

occurs in this ecosystem to preserve inorganic N preferentially in the NH_3 form. Taken these findings together, it is likely that not only the presence of ammonia oxidizers is fundamental for nitrification to occur, but that the microbial community composition and diversity affects the direction in which N process occur in soil. Most possibly there is a correlation between abiotic and biotic conditions that limits the abundance of autotrophic ammonia oxidizers, as for example the competition for NH_4^+ by plants or heterotrophic microbes or through dissimilatory reduction of NO_3^- to NH_4^+.

General Index

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Motivação

Micro-organismos aprovisionam diversos serviços ecossistêmicos, tais como a reciclagem de nutrientes e a decomposição de matéria orgânica, a reciclagem de dejetos e o controle biológico de pestes. Na economia mundial atual, esses serviços representam um terço da contribuição anual dos serviços ecossistêmicos terrestres, significando uma média global estimada de 1,6 trilhões de dólares por ano. Além disso, a interação entre micro-organismos e plantas, especialmente na rizosfera, é responsável pela nutrição e saúde das plantas, que dependem de reações catalisadas pelos micro-organismos no solo. Consequentemente, o crescimento populacional mundial depende do fornecimento de comida pela agricultura e pecuária, por sua vez condicionado à reciclagem de nutrientes por micro-organismos no solo.

Todavia, o objetivo dos micro-organismos é o de obter energia para seu próprio metabolismo ou produção de biomassa. Por sua vez, se o substrato é provido em excesso no ambiente, uma maior concentração de produtos será liberada e não incorporada à biomassa microbiana (e de plantas). Por exemplo, o uso de fertilizantes nitrogenados em excesso na agricultura pode potencialmente levar ao aumento de emissão de gases nitrogenados causadores do efeito estufa (N_2O), e também às perdas de nitrato que levam à contaminação de cursos de água.

A aquisição de energia em solos não é tarefa simples: formas de vida diferentes competem para a viabilidade de substratos, ou também colaboram para a troca de substrato/produto. Essa competição acontece a todo momento, em micro *hotspots* do solo; uma batalha entre plantas e micro-organismos e entre diferentes micro-organismos.

No último dezembro, na COP 21, a maior parte dos países concordou que devem ser tomadas ações para a redução do aquecimento global, que está notadamente associado à emissão de gases de efeito estufa. No entanto, os micro-organismos não foram protagonistas nas discussões da reunião acima citada, apesar de estarem diretamente relacionados à capacidade de um ambiente de ser fonte ou captador dos gases de efeito estufa. Nesse contexto, a ecologia microbiana de solo tem como foco a identificação de genes que controlam especificamente as funções relativas à emissão desses gases ou outras vias metabólicas da ciclagem de nutrientes. Desta forma é possível monitorar as mudanças no ecossistema e aquelas relativas aos serviços ecossistêmicos providos pelos micro-organismos.

Mudanças climáticas, pluviometria e o regime de fogo devem ser considerados nos estudos da savana tropical sazonalmente seca no Brasil Central. Esta, o Cerrado, é a savana de maior biodiversidade, e em grande parte endêmica. Ainda mais, sua área está em constante modificação devido à fronteira agrícola, envolvida na produção de *commodities* brasileiras.

Tal como referido anteriormente, as comunidades microbianas são os atores das transformações bioquímicas que ocorrem nos solos, e as técnicas moleculares são usadas para descrever e compreender as modificações que ocorrem nas comunidades microbianas de acordo com as mudanças no ambiente amostrado. Nesse contexto, e para nosso conhecimento, esta tese é o primeiro trabalho que considera a variação dos grupos funcionais relacionados à ciclagem do N no Cerrado, medida pela abundância de genes microbianos no solo.

Objetivos e hipóteses

		Objetivos	Hipóteses
Gerais		Descrever a microbiota do solo do Cerrado nativo e convertido à plantação da soja, fazendo uso de técnicas moleculares e com foco no metabolismo do N Estudar as baixas taxas de nitrificação líquida observadas nesses solos ao analisar a comunidade microbiana Analisar a relação entre biodiversidade microbiana e a provisão de serviços ecossistêmicos como a ciclagem de nutrientes Determinar o impacto da agricultura na abundância de oxidantes de amônia e seu funcionamento	A comunidade de oxidantes de amônia será menos abundante nos solos do Cerrado, considerando as baixas taxas de nitrificação A abundância relativa dos genes relativos ao ciclo do N irá variar conforme as qualidades físico-químicas dos solos amostrados O solo agrícola apresentará uma estrutura diferente da comunidade de oxidantes de amônia em relação ao solo nativo
Específicos	**Capítulo 2**	Analisar a diversidade taxonômica e funcional dos micro-organismos do solo do Cerrado, usando dados de metagenômica Identificar genes dos grupos microbianos responsáveis pelo metabolismo do N Estabelecer a correlação entre a abundância relativa dos genes do metabolismo do N e as características do solo e da vegetação entre e dentro dos parques de conservação	A comunidade microbiana irá diferir de acordo com a distância biogeográfica e as características físico-químicas dentro e entre os parques de conservação A abundância dos genes anotados para o metabolismo do N irá refletir a razão C:N, o pH, o teor gravimétrico de água e os conteúdos de N e C dos solos amostrados dentro e entre os parques
	Capítulo 3	Investigar a variação temporal e espacial da abundância de archaeas e bactérias oxidantes de amônia por PCR quantitativa ao longo do cultivo da soja no bioma Cerrado Elucidar as variáveis físico-químicas que explicam a mudança na abundância dos oxidantes de amônia	A razão entre archaeas e bactérias oxidantes de amônia irá modificar ao longo do cultivo da soja devido ao aumento do pH e à adição de fertilizantes nitrogenados A comunidade de archaeas oxidantes de amônia será maior em número que aquela de bactérias no solo nativo de Campo sujo e na área de manejo da soja durante o pousio devido ao pH mais ácido e à provisão de NH_4^+ principalmente por mineralização

Capítulo 4	Incubar solos em microcosmos para testar o efeito da água e do pH na habilidade do solo de acumular nitrato Testar o potencial biológico de inibição para nitrificação em solos do Cerrado contra um solo exótico agrícola com alta capacidade de nitrificar	Exsudatos naturais de algumas plantas estarão relacionado à potencial inibição biológica e, portanto, à redução do crescimento e atividade de oxidantes de amônia O baixo teor gravimétrico de água e o baixo pH dos solos do Cerrado limitará o crescimento e atividade de oxidantes de amônia

Organização de capítulos

Capítulo 1 – Introdução apresenta a revisão da literatura quanto ao conhecimento dos processos enzimáticos microbianos envolvidos na ciclagem de nitrogênio em solos, com uma perspectiva direcionada ao bioma Cerrado, que é o foco desta tese

Capítulo 2 – Análise metagenômica da microbiota do solo de Cerrado nativo, com especialmente interesse na abundância relativa de genes anotados para o metabolismo do nitrogênio

Capítulo 3 – Cultivo de soja na Fazenda Tabapuã dos Pirineus pela primeira vez foi escolhido para investigar o efeito a curto prazo do manejo agrícola sobre a abundância de archaeas e bactérias oxidantes de amônia

Capítulo 4 – Limitação da oxidação de amônia em solos do Cerrado foi avaliada em microcosmos e culturas puras para testar o efeito da água, pH e potenciais inibidores biológicos produzidos por plantas sobre a nitrificação

Capítulo 5 – Discussão com o objetivo de retornar aos principais pontos apresentados nos capítulos anteriores e também estabelecer novas considerações sobre regulações bióticas e abióticasdo ciclo do nitrogênio e, mais especificamente, da nitrificação, que ocorrem nos solos

Capítulo 6 – Conclusão

Chapter 1 – N cycle, nitrification and the Cerrado biome: literature review [1]

"We became scientists because we are curious – we are driven to solve the puzzles that nature presents."

Joshua Schimel

Nitrogen cycling is mainly controlled by microorganisms in a multitude of processes and regulations. Advances in research presents novelties that were sometimes anticipated based in N thermodynamics. For example, just recently it has been discovered the "comammonas" process, which is the ability of ammonia oxidation to nitrite and subsequently to nitrate in a same organism; metabolism predicted by the higher gain of energy when are substrate- and spatial-limited (Daims *et al.*, 2015; van Kessel *et al.*, 2015).

Plants and microorganisms can assimilate N in the form of ammonia, nitrate, and sometimes organic N, or N_2 for a few *Bacteria* and *Archaea*. N_2 enters the lithosphere and is biologically transformed to NH_4^+. In turn, NO_3^- is made available by the dissimilatory process of ammonia oxidation by autotrophic *Archaea*, *Bacteria* or heterotrophic *Bacteria* and *Fungi*. Microorganisms compete within themselves and with plants to use the NO_3^- available in soil, which can be either assimilated or used as electron donor. The balance of processes

[1] A modified version of this thesis introduction and discussion will be submitted as a review on the N cycle of the Cerrado soils.

and the soil conditions determines the availability of N returning to NH_4^+ or being completely reduced to N_2.

N is essential to primary productivity and, in nature, is mainly dependent on biological nitrogen fixation, which produces reactive N. On the other hand, the non-natural chemical conversion of atmospheric N_2 to NH_3 in the Haber-Bosch process, increased the reactive N concentration in the environment, presenting consequences due to N loss to the atmosphere as N trace gases, or to water courses as nitrate produced during nitrification (Galloway and Cowling, 2002).

Nitrification

Nitrification can be measured as gross or net rates; the first is quantified by the assimilatory or dissimilatory processes calculated for example by the ^{15}N pool dilution methods (Davidson *et al.*, 1991). Net nitrification is obtained by the variation of NO_3^--N concentration in incubated soil (either in laboratory or field conditions) during an established period of time. However, only the first method can assess if $^{15}NO_3^-$ pool is diluted with $^{14}NO_3^-$ produced by autotrophic nitrifiers from $^{14}NH_4^+$ or by heterotrophic organisms from organic ^{14}N (Davidson *et al.*, 1991). Net nitrification in native undisturbed Cerrado soils is low and sometimes undetectable. These soils present high NH_4^+-N:NO_3^--N ratio (Nardoto and Bustamante, 2003) and insignificant N_2O emissions (Cruvinel *et al.*, 2011; Pinto *et al.*, 2006; Pinto *et al.*, 2002). Thus, the investigation of nitrification in the Cerrado biome is of particular interest for its N-limitation (Araujo *et al.*, 2012), with higher rate of N immobilization than mineralization (Nardoto and Bustamante, 2003), which leads to a need of fertilizers and liming when land use is changed for agriculture.

Cerrado is the savanna of Central Brazil and, as such has a plant cover distribution dependent on the interaction between water and nutrient availability (Medina, 1987) in (Bustamante *et al.*, 2006), with weathered soils with low nutrient availability (Reatto *et al.*, 1998). The Cerrado presents a range of herbaceous and tree/shrub strata from grassland to savanna and forest formations, that are related to the type of soil (Reatto *et al.*, 1998), which may present varying contents of nitrogen according to the tree-shrub layer density, the fire regime and the land use change (Bustamante *et al.*, 2006).

Bustamante *et al.* (2006) reviewed N concentration and N dynamic in ecosystem compartments for the tropical savannas, but it remains to be discussed the microorganisms

associated with N metabolism. Microbial ecology has been used in the last couple of decades to improve knowledge on biogeochemical processes in the environment. The presence of genes can be directly measured by PCR quantification or the taxonomic categories can be assigned through sequencing (Figure 1). Metagenomics' studies are primer-independent thus allowing a more holistic description of genes abundance in the ecosystem. Nevertheless, the current culture-independent methods depend on database search and a great number of genes is still unclassified. Therefore, classical microbiology approach with isolated microorganisms is complementary.

Nitrification involves two groups of specialized organisms phylogenetically unrelated: the ammonia-oxidizers and the nitrite-oxidizers. The oxidation of ammonia is often the focus of research, because it is the limiting-step for nitrification to occur. However, and as mentioned above, in the end of 2015 two groups were able to identify an organism, "*Candidatus* Nitrospira inopinata able to perform the complete oxidation of ammonia to nitrate, isolated from a biofilm in a pipe under hot water flow (Daims *et al.*, 2015) and from an ammonium-oxidizing biofilm from an aquaculture system filter (van Kessel *et al.*, 2015).

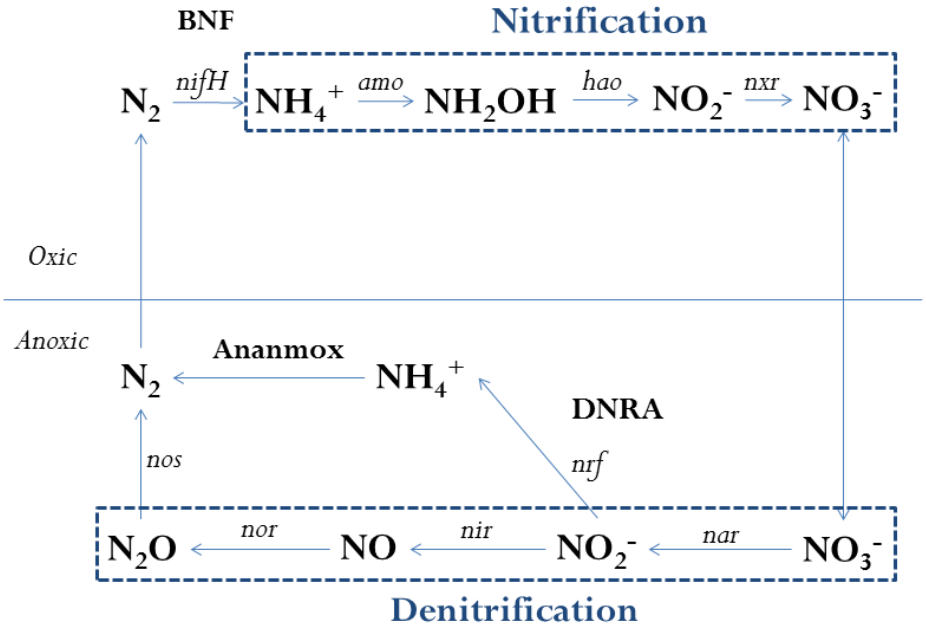


Figure 1. N cycle processes in oxic and anoxic environments. Special highlight to nitrification and denitrification. In italic genes often used to quantify these processes BNF: Biological nitrogen fixation. DNRA: dissimilatory nitrate reduction to ammonium. Ananmox: anaerobic oxidation of ammonia.

The presence of ammonia oxidizers is quantified by the *amoA* gene coding for the subunit A of the enzyme ammonia monooxygenase, and it is catalyzed by autotrophic *Bacteria* (AOB) – *Nitrosomonas* (β-proteobacteria), *Nitrosospira* (β-proteobacteria) (Kowalchuk and Stephen, 2001) and *Nitrosococcus* (γ-proteobacteria) – or autotrophic *Archaea* (AOA), phylum Thaumarchaeota. The ammonia monooxygenase is a protein of membrane that converts NH_3 to NH_2OH (hydroxylamine, HAO), then released into the periplasm to be oxidized by HAO to NO_2^- in AOB (De Boer and Kowalchuk, 2001). No *hao* gene has been detected in AOA genomes, but *N. maritimus* seems to produce NH_2OH, possibly through a different enzyme complex (Vajrala *et al.*, 2013). AOA and AOB appear to be mechanistically similar even though differ, within other things, in the dependence of copper (AOB) rather than iron as the redox active for AOA (Stahl and de la Torre, 2012) and in the organization of the operon AMO. In AOB the AMO operon has a conserved organization as *amoCAB* (Bothe *et al.*, 2000; Nicol G.W., 2006; Norton *et al.*, 2002), while in Thaumarchaeota the organization as *amoAxCB* varies between lineages (Bartossek *et al.*, 2012; Blainey *et al.*, 2011).

The majority of studies with soils show AOA as more abundant than AOB and more frequently associated with nitrification rates (Leininger *et al.*, 2006; Mao *et al.*, 2011; Prosser and Nicol, 2012). In addition, AOA seem to prefer ammonia generated from the mineralization of organic N and are the predominant ammonia oxidizers in acidic soils (Levičnik-Höfferle *et al.*, 2012; Prosser and Nicol, 2012; Zhang *et al.*, 2012) or in environments with little availability of NH_4^+ (Gubry-Rangin *et al.*, 2011; Gubry-Rangin *et al.*, 2010; Nicol *et al.*, 2008). This apparent niche differentiation (Prosser and Nicol, 2012) might be important to consider in view of the economic and ecological costs of fertilization and nitrogen losses.

Heterotrophic nitrification

The structure and functioning of ammonia oxidation in heterotrophic nitrifiers is not as well described. Most of the heterotrophic nitrifying bacteria have similar enzymes as the autotrophic counterparts as reviewed in (De Boer and Kowalchuk, 2001); and the *amoA* gene is at least partially homologue to that of *N. europaea* (Bothe *et al.*, 2000). Nevertheless, the broad range of phylogenetic heterotroph bacteria able to nitrify complicates the use of a molecular assay to determine their presence in the environment. Furthermore, their potential metabolic activities do not ensure the contribution to the N metabolism (Kowalchuk and Stephen, 2001), since heterotrophic nitrifiers can use organic

or inorganic N, but ammonia oxidation is not linked to cellular growth as in autotrophs. The presence of heterotrophic nitrifiers in soil can be presumed by the accumulation of nitrate in soils incubated with acetylene, a specific inhibitor of autotrophic nitrification, as suggested for Cerrado soils (Poth *et al.*, 1995). On the other hand, nitrification in *Fungi* seems to involve the reaction of N compounds with hydroxyl radicals formed potentially during cell lysis or lignin degradation (De Boer and Kowalchuk, 2001).

Moreover, some of the bacteria able to perform nitrification heterotrophically can combine nitrification-denitrification processes; where denitrification is used by the organism to dissipate reducing equivalents (NADH) under low oxygen conditions, allowing a greater growth rate on an environment with substrate in excess (De Boer and Kowalchuk, 2001) , which is less likely in Cerrado soils.

Denitrification

Denitrification alone is represented as the reduction of NO_3^- to NO_2^-, and subsequently to NO, N_2O and N_2 by the same organism or more commonly by different organisms, thus considered a modular process (Graf *et al.*, 2014). The reduction of NO_2^- to NO is catalyzed either by a copper-containing enzyme, that can be identified by the measurement of the gene *nirK* abundance; or the nitrite reductase encoded by *nirS* which is a cytochrome cd1 (Mohan *et al.*, 2004). These are dissimilatory enzymes associated with electron transport phosphorylation. However, nitrite reductases can also be assimilatory when the reduction of NO_2^- leads to NH_4^+. These use reduced pyrimidine nucleotides or ferredoxin as electron donor: the cytoplasmatic NirB is more common in fermentative bacteria and the periplasmic nitrite reductase, deduced by the presence of the gene *nrfA* in the environment, is found in a wider range of bacteria than the above (Mohan *et al.*, 2004).

Denitrification is dominant on nitrate-rich environment with low electron donors' concentration; however, NO_3^- and NO_2^- reduction to NH_4^+ predominates on an electron-rich environment where NO_3^- is in low concentration. Dissimilatory nitrite reduction to ammonium (DNRA), known also as fermentative reduction of nitrate or ammonification, is the concurrent process to denitrification, representative in reduced and C-rich environments. Available soil literature is smaller for DNRA than denitrification, even though DNRA is also a process widespread among bacteria (Mohan *et al.*, 2004). DNRA was suggested as a short-circuit of N cycle, returning NO_3^- to NH_4^+ (Cole and Brown, 1980), and despite not frequently considered in terrestrial experiments, since it is an anaerobic

process, it can be relevant in soils (Rütting *et al.*, 2011). On the other hand, anammox, the anaerobic ammonia oxidation to N_2 seems to be strictly present in anoxic environments.

Denitrifiers guilds and N trace gases emission

A microbial guild is described as a group of organisms occurring in the same space and time, and that use same resources (Fauth *et al.*, 1996). The relative abundance of different microbial guilds is dependent on soil characteristics. An increase in soil water content after the first rains that follow the dry season in the Cerrado promoted higher mineralization (Nardoto and Bustamante, 2003), reflecting a higher microbial activity and nitrification (da Silva, 2004). More specifically, AOA and AOB differ in their niches in soil according with different pH and ammonia availability. Similarly, denitrifier's guilds, meaning organisms containing either *nirK* or *nirS* genes, also respond differently to the environment (Enwall *et al.*, 2010; Jones and Hallin, 2010) as well as the *nosZ* organisms from clade I or II (Jones *et al.*, 2013). The ratios of *nirS*/*nirK* type and *nosZ* clade I/clade II are related and have an effect on the soil N_2O sink capacity, more significant in environments dominated by *nosZ* clade II (Jones *et al.*, 2014).

In turn, the balance between the processes described above controls N trace gases emissions (Conrad, 1996). Emission of NO and N_2O can occur either during nitrification or denitrification. A special attention is given to agricultural fields as fertilization increases the microbial transformation of reactive N (Galloway and Cowling, 2002). N_2O is a significant greenhouse gas after CO_2 and CH_4, and is also a relevant ozone depleting gas (Ravishankara *et al.*, 2009) when oxidized to NO, as reviewed recently (Kanter *et al.*, 2013). In addition, N oxides (NO and NO_2) are removed from the troposphere as nitric acid, contributing to ecosystems acidification.

Emissions of nitric oxide (NO) represents 0.4 kg N ha^{-1} $year^{-1}$ loss of N in the Cerrado (Bustamante *et al.*, 2006) and is emitted in higher concentration than N_2O in those soils, as expected by the dry and well-aerated characteristic of these soils (Pinto *et al.*, 2002). The "hole-in-the-pipe" concept states that soil water content is the principal control on the balance of production, consumption and diffusive transport between NO, N_2O and N_2 in soils (Davidson *et al.*, 2000). The ratio of emission between N_2O and NO should be 1 in soils with water filled pore space (WFPS) at 60% (Davidson *et al.*, 2000). Pinto *et al.* (2002) also emphasized that soil moisture and vegetation were more strongly associated with NO emission than fire regime. In addition, N availability influences both gases emission,

however, in a wet soil N_2O is more prevalent and the analysis of only NO would lead to false conclusions that nitrogen availability does not matter (Davidson *et al.*, 2000).

Cerrado soils can experience short moments of flooding during the first rainfall after the dry season, but they are often described as well-drained, leached and oligotrophic soils (Ribeiro and Walter, 2008). The first rains after the dry season promote an increase of 100 fold on the emission of NO in the Cerrado, which does not continue during the wet season (Pinto *et al.*, 2002). Although denitrification can occur in aerated soils (Braker *et al.*, 2015), it is not expected in the Cerrado soils, especially because of the low accumulation of NO_3^- in these soils, and the dominance of N form as NH_4^+ is associated with low N trace gases emission (Davidson *et al.*, 2000).

N fixation and other sources of N

Reactive N enters the system through biological or chemical N fixation, which is the conversion of the inert gas N2 to NH_4^+. In the Cerrado soils, the biological nitrogen fixation (16 a 44 kg N ha^{-1} $year^{-1}$) exceeds the abiotic fixation through electrical discharges (4 kg N ha^{-1} $year^{-1}$) (Bustamante *et al.*, 2006; Cleveland *et al.*, 1999). This important source of N is possibly related with the high abundance of plant species from the Fabaceae family in the Cerrado (Filgueiras, 2002), even though very few studies have focused on the nodular activity of these plants (Bustamante *et al.*, 2012c). It is recognized though that O_2, P, Ca and Al concentrations, soil moisture, bacterial density and plant needs of N determine the ability of nodulation by symbiotic dyazotrophs (Bustamante *et al.*, 2006). As well as for denitrifiers, the genes encoding the enzyme nitrogenase (*nifH* being the gene used to quantify N fixation) are widespread in the *Bacteria* and *Archaea* domains. Although nitrogenase is an enzymatic complex sensible to oxygen, dyazotrophs are not necessarily anaerobic (Falkowski *et al.*, 2008).

Organic matter mineralization recycles N in soils, which can then be assimilated by plants and microorganisms, or lost via NH_3 volatilization, enzymatic denitrification and NO_3^- leaching as discussed above. These losses depend on climatic and edaphic conditions, but, in general, increase with land use change. Volatilization of ammonia increases with soil alcalinization, leaching with increased nitrification and consequently higher substrate for denitrification and emissions of N trace gases. Despite the fact that the Haber-Bosch method allowed the increase for food production, there were consequences to the ecosystem functioning, as N_2O is a greenhouse gas, NO catalyzes the ozone layer

destruction, and nitrate causes eutrophication in water courses due to increased leaching. Furthermore, only half of N added in crops is used by the plants (Galloway and Cowling, 2002).

NO_3^- is considered the main form of nutrition used by plants in well aerated soils, where nitrification is more prone to happen. However, in Cerrado soils, the greatest part of inorganic N is found in the form of ammonia (Nardoto and Bustamante, 2003), suggested to be related with the low pH found in these soils, or competition between plants and microorganisms. Furthermore, the availability of inorganic N in soil depends on organic matter mineralization, which is lower than N immobilization in Cerrado soils (Nardoto and Bustamante, 2003). For example, some forests in their climax are more efficient in N use, potentially by inhibiting nitrification, and so maintaining predominantly ammonia than nitrate in the soil solution, which leads to lower losses of N as reviewed recently (Subbarao *et al.*, 2015). Therefore, the observation of dynamics between plants and microbial community in the belowground can help understand the balance in N transformations and N retention and therefore provide a model for a more sustainable crop productivity.

Land use impact on microbial communities

Brazil is the fourth worldwide country in agriculture production, which depends on inorganic fertilizers. The progressing frontier of agriculture and managed pasture for cattle breeding promoted the change of approximately 53% change of the Cerrado's original area (Beuchle *et al.*, 2015). Soybean, maize, cotton and sugarcane stand out as the major crops cultivated in the Cerrado region, in which only the first is partially independent on the addition of fertilizers (Mendes *et al.*, 2003). A study published in 2010 showed that 81% of exported soybean was produced in Brazil, EUA and Argentina together. This reflects a global trade of biogeochemical N cycling represented in 25% by the soybean commodity (Lassaletta *et al.*, 2014).

Land use impacts soil microbiota and consequently the terrestrial ecological services it provides (e.g. decomposition and nutrient cycling), it modifies C and N dynamics (Bustamante *et al.*, 2012c), and it changes C and N stocks and sink and greenhouse gases emission (Carvalho *et al.*, 2009). In turn, the alteration in N dynamics leads to a reduction of biodiversity (Bustamante *et al.*, 2012c; Jacobson *et al.*, 2011), facilitates the invasion by exotic species (Lannes *et al.*, 2012) and modifies decomposition and nutrient cycling (Kozovits *et al.*, 2007).

Then again, governmental initiatives are also concerned with preserving the Brazilian biomes biodiversity in conservation unities. However, only 2.2% of this biome is under unities of integral protection and other 1.9% in unities of sustainable use (Klink and Machado, 2005; Marris, 2005) ensuring its status of a hotspot for biodiversity conservation (Myers *et al.*, 2000). In this context, it is important to have in mind that soil is the main actor in the ecosystem conservation, especially in seasonally dry environments as the Cerrado, where climatic change will probably modify rain distribution and regime, changing also the fire frequency and potentially nutrients and biomass loss (Bustamante *et al.*, 2012c).

More specifically, soil microbial diversity contributes to the resistance/resilience of the system, which means that the lower diversity after land use change can alter the stability of the ecosystem. Mao *et al.* (2011) observed that N fertilization for bioenergy crops (*Zea mays* and *Miscanthus giganteus*) altered the microbial communities, and induced the modification on 15% to 30% of the relative abundance of nitrification and denitrification genes. This is an example of how agriculture impacts microbial potential ecological functions. Same results were observed in the Cerrado soils, where soybean cultivation reduced microbial N independent on the soil management or the plant growing stage in comparison to a soil under native Cerrado (Perez *et al.*, 2005). Moreover, land use management in the Amazonian forest changed the composition and abundance of soil microbial communities, related with the modification in soil pH and OM (Paula *et al.*, 2014).

Despite the known functionality of soil microbiota regulating fertility and health by decomposing organic matter and through biogeochemistry we still have a lot to understand from microbial patterns of distribution in terrestrial ecosystems. Free-living microorganisms also present patterns of biogeography (Martiny *et al.*, 2006), and should be included in models for biome sustainability specially in biomes threatened as the Cerrado. As discussed, these correlations of change in soil characteristics and microbial communities can be monitored targeting specific genes with molecular techniques that complement ecological and edaphic research in the quest to value ecosystems services provided by soil biota. This work main objective is to link soil characteristics with N cycling dynamics and the microbial functional potential in the Cerrado biome, as a step to identify key drivers of sink/source of N in those soils, and allow further incorporation of biological drivers into predictive models.

Chapter 2 – Distribution of microbial communities in two Cerrado conservation parks with a metagenomics approach, with special focus on the N metabolism [2]

"everything is everywhere" and why do we care

Baas Becking

Abstract

Nitrogen is the base for primary productivity, and primarily cycled by the soil microbiota. Climate change, land use side effects and nitrogenous fertilizers employment are changing the global N budget. The Cerrado is the largest savanna in South America, and as others savannas in the world, is suffering from the land use change to agriculture and pasture. Yet, little is known of how these changes affect soil microbial communities. Undisturbed areas are essential to understand the natural processes rates that occur in soil. We used shotgun metagenomics to compare the functional attributes of N cycling from the soil microbiota present in two parks for conservation of the Cerrado biome, 500-km distant from each other, with varying altitude, soil texture and water content. Types of vegetation sampled within each park masked the altitude and distance effects. The soils with greater and lower soil water content presented the highest levels of α-diversity, which may relate with greater evenness of species to overcome a less enabling environment. N fixation, nitrosative stress and ammonification from nitrate and nitrite differed significantly between the sites sampled. Across all soils, the assimilation of ammonia and ammonification were the most abundant subsystem of nitrogen cycle, corroborating the Cerrado literature that states ammonia as the main nitrogen form. We detected genes for denitrification enzymes, but only two hits for the nitrification process were described. This study suggests that the N cycle processes occurs differently between the sites. Furthermore, we suggest that each type of vegetation is relevant for N conservation in this biome.

Keywords: Brazilian savanna, Cerrado, N cycle, ammonia, nitrification, denitrification

[2] A modified version of this manuscript will be submitted to publication, possibly including a discussion on the cycle of C and S.

Introduction

Savanna ecosystems hold almost one fifth of the world's population. Cerrado is the main tropical savanna in the south hemisphere. It is a representative biome in central Brazil, the second largest in South America, and a wildlife corridor for species from the Amazon and Atlantic rainforests. As others savannas, Cerrado is controlled by the interaction between water and nutrient availability (Bustamante *et al.*, 2006). Cerrado has an alternating wet and dry seasons and fire frequency that might change attributable to the global climatic changes, as higher temperatures, decreased rainfall and longer dry season may have an impact on net ecosystem exchanges and reduced nutrient stocks (Bustamante *et al.*, 2012c).

The Cerrado is characterized by a continuous herbaceous layer over which stands a discontinuous tree/shrub stratum, resulting on a range of ecosystems from grassland to savanna and forest formations. This variation on the types of vegetation found in the Cerrado biome is related to the type of soil, mostly weathered with low nutrient availability (Reatto *et al.*, 1998), which may present varying contents of nitrogen according to the tree-shrub layer density, the fire regime and the land use change (Bustamante *et al.*, 2006). Plant type and soil texture influence microbial community structure in the rhizosphere soil (Tkacz *et al.*, 2015).

Due to its progressively land use change - approximately 53% of the Cerrado landscape has been transformed (Beuchle *et al.*, 2015) – the Cerrado is considered a hotspot for biodiversity conservation (Myers *et al.*, 2000), and approximately 2% of this biome is under protection (Marris, 2005). However, conservation unities designated for environment protection are not necessarily continuous (Beuchle *et al.*, 2015). In addition, a special attention for conservation is paid to forest formations bordering water courses in the Brazilian legislation. Nevertheless, ecological insurance theory assumes that a better occupation of space by higher diversity leads to a better system productivity (Yachi and Loreau, 1999), i.e. the distribution of Cerrado in different vegetation patches. Similarly, we suggest that microbial community performs differently in these patches, due to the different resources and soil characteristics.

The relative abundance of microbial phylogenetic groups varies according to Cerrado types of vegetation, i.e. savannas grassland and shrubland or riverbank (Araujo *et al.*, 2012; Catão *et al.*, 2013; de Castro *et al.*, 2008; Quirino *et al.*, 2009). Soil pH is directly

linked to nutrient availability in soil and is often associated with the distribution of bacterial communities in soil (Bru *et al.*, 2011; Griffiths *et al.*, 2011; Kuramae *et al.*, 2012; Rousk *et al.*, 2010) (Lauber *et al.*, 2009). However, in the Cerrado soil moisture is more strongly related with microbial community structure (Catão *et al.*, 2014; Pereira de Castro *et al.*, 2016; Viana *et al.*, 2011), which can be associated with soil texture and its water retention capacity. Recently, Pereira de Castro et al. (2016) discussed the general metabolic potential distribution in the Cerrado biome besides the taxonomy approach. Nonetheless, until now no work has focused on the microbial genes associated with nitrogen cycling in the Cerrado, despite the need to understand microbial governed N pathways in undisturbed ecosystem and the use of high- throughput shotgun sequencing to characterize the N metabolism in other environmental samples (Andreote *et al.*, 2012; Cobo-Díaz *et al.*, 2015; Pfister *et al.*, 2010).

Nitrogen is mainly recycled in soils through nitrogen fixation, SOM mineralization, ammonification, nitrification and denitrification. In undisturbed ecosystems, N leakage is minimized, and nitrification is restricted, but little is understood about this in the Cerrado biome. The ecology of N dynamics between compartments has been reviewed beforehand for this biome (Bustamante *et al.*, 2006), which is characterized by a high NH_4:NO_3 ratio, low nitrification and low N gas emission.

This work was conducted to investigate the variation of relative abundance of taxonomic and functional potential genes in the soil of Cerrado. It was considered the range of types of vegetation found in two 500-km distant parks of conservation with different altitudes, and pluviometry. The first hypothesis assumes that vegetation and edaphic characteristics, which vary within and between parks, will reflect on the microbial diversity, due to different resource use or environment constraints. Secondly, we hypothesized that genes related to N metabolism would vary with the soil characteristics specific to each vegetation type as carbon and NH_4^+ availability, pH and soil moisture. To test these, 24 metagenomes (eight areas in triplicates) were sequenced to describe the functional and taxonomic categories of Cerrado soils microbiota at a regional scale. We believe that microbial controls of N conservation - the balance of assimilative and dissimilative processes - in the Cerrado soils can help future works of biogeochemical models or soil management improvement.

Material and methods

Soil sampling and physicochemical analyses

This study was performed in two sites: the National Park of the Chapada dos Veadeiros (PNCV) and State Park of Serra Azul (PESA) both located in Central Brazil (Figure 9), classified as the Cerrado biome and approximately 500 km distant (coordinates provided in Table S1). The two sites diverge in altitude (Table S1). The climate of these regions is classified as Koppen Aw and the annual mean rainfall is of 1500 mm mostly during the rainy season, which happens from October to May. Sampling was performed at the end of the rainy season: the accumulated rain and the mean temperature from the month of April (2013) until the sampling day in the PNCV was of 2.2 mm and 21 °C; for the PESA no rain was measured on the month of May (2013) and an average 27 °C were measured. In total 8 areas and 6 different vegetation types were sampled.

In the PNCV and the PESA, beside some other parks, it was installed modules of standardized sampling thanks to the project financed by CNPq "Diversidade biológica do Cerrado: estruturas e padrões".. These modules were created within the "Rede ComCerrado" (Portaria MCT 319, 7 May 2009), which is a network founded by several research groups from public institutions in Brazil to monitor Cerrado's biodiversity. These modules establish 5 km^2 area bordered by 2 lines oriented east-west 1 km apart and 5 km long as standardized in the literature to sample extensive biomes as the Amazon rainforest (Magnusson *et al.*, 2005). Along these 5 km, 10 parcels (5 in each line) were established, one per km and a perpendicular line of 250 m was draw along the terrain level curve (Figure 9C).

Soil was sampled from a total of 24 points (8 sites in triplicates from the upper 10 cm. Replicates in each site were taken approximately 50 m apart (at 50, 100 and 150 m inside the parcel line (red line in Figure 9C), soil was sieved through a 2-mm mesh and stored on ice upon collection and on -20°C in the laboratory before physicochemical and molecular analysis. Soil texture and content of macro and micronutrients were measured by using standard methods (Soils Embrapa–SNLCS) at SoloQuímica, Inc, Brasília, Brazil. Inorganic N was determined as described previously (Catão *et al.*, 2016).

The PNCV was created in 1981 and includes the municipal areas of Alto Paraíso de Goiás, Cavalcante and Colinas do Sul (state of Goiás) (MMA, 2011). Soils are poor in nutrients and, with varying types of soil, as Neossolos litólicos (Entisol, Udorthent), Plintossolos (Oxisol), Cambissolos (Inceptisol), hydromorphic soils and Latossolos (Oxisol) (Haridasan,

2007). In the PNCV, soil samples were obtained in a Cerrado *sensu stricto* (SS), in a riverbank gallery forest, hereafter called "Mata de galeria" (MG), a flooded grassland, hereafter named "Campo limpo" (CL) and a Cerrado "rupestre" (CR), (Figure 2). The physicochemical variables observed in the sampled soils are described in Table 1.

Table 1. Physicochemical variables (mean ± SE) of the sampled sites in PNCV

Park	National Park of the Chapada dos Veadeiros			
Type	MG	CL	SS	CR
SWC (% H_2o g^{-1} DS)	45.8*	48.1 ± 8.8	17.8 ± 1.8	6.0 ± 0.6
Clay (g kg^{-1})	233 ± 8	167 ± 22	333 ± 22	133 ± 8
Sand (g kg^{-1})	617 ± 22	758 ± 22	608 ± 17	842 ± 8
Silt (g kg^{-1})	150 ± 29	75 ± 14	58 ± 8	25 ± 0
pH in H_2o	5.70 ± 0.06	5.27 ± 0.34	4.93 ± 0.09	5.00 ± 0.06
pH in KCl	3.97 ± 0.12	4.20 ± 0.06	3.73 ± 0.03	3.70 ± 0.06
P (mg dm^{-3})	10.83 ± 2.88	4.73 ± 0.66	1.17 ± 0.19	2.53 ± 0.20
Ca ($cmol_c$ dm^{-3})	1.10 ± 0.45	0.57 ± 0.12	0.50 ± 0.06	0.57 ± 0.15
Mg ($cmol_c$ dm^{-3})	0.53 ± 0.09	0.30 ± 0.00	0.33 ± 0.03	0.30 ± 0.00
K ($cmol_c$ dm^{-3})	0.17 ± 0.02	0.02 ± 0.01	0.09 ± 0.01	0.04 ± 0.00
Na ($cmol_c$ dm^{-3})	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
CTC ($cmol_c$ dm^{-3})	9.33 ± 0.33	6.00 ± 0.58	7.67 ± 0.33	5.67 ± 0.33
Al ($cmol_c$ dm^{-3})	2.33 ± 0.52	0.90 ± 0.15	1.80 ± 0.15	1.00 ± 0.10
H+Al ($cmol_c$ dm^{-3})	7.27 ± 0.64	5.13 ± 0.35	6.53 ± 0.17	4.43 ± 0.38
C (g kg^{-1})	182.07 ± 51.98	47.17 ± 13.20	29.33 ± 2.60	11.97 ± 1.24
OM (g kg^{-1})	313.17 ± 89.42	81.13 ± 22.68	50.43 ± 4.47	20.60 ± 2.15
B (mg dm^{-3})	0.63 ± 0.06	0.70 ± 0.01	0.69 ± 0.07	0.58 ± 0.10
Cu (mg dm^{-3})	0.13 ± 0.04	0.19 ± 0.04	0.17 ± 0.02	0.15 ± 0.03
Fe (mg dm^{-3})	72.47 ± 16.51	158.33 ± 9.02	246.00 ± 54.99	136.27 ± 27.79
Mn (mg dm^{-3})	5.68 ± 0.88	3.31 ± 0.10	3.41 ± 0.12	2.94 ± 0.03
Zn (mg dm^{-3})	0.85 ± 0.18	0.52 ± 0.17	0.40 ± 0.03	0.19 ± 0.02
S (mg dm^{-3})	25.03 ± 8.79	43.30 ± 7.40	12.70 ± 2.04	10.43 ± 1.21

SWC: Soil water content. *Measurement from only one sample. MG: Mata de galeria; CL: Campo limpo; SS: Cerrado *sensu stricto*, CR: Cerrado rupestre

The PESA is located entirely in the municipal area of Barra do Garças (state of Mato Grosso) and occupies 11,002.4 ha, in which the topography can vary (350-730 m). Soils are predominantly Litólicos (Udorthent) and Latossolo amarelo (Oxisol, Udox) (in the plain areas). PESA was created in 31 May 1994, accordingly with the State Law of Matogrosso 6.439. More about the vegetation types in this park can be found in the literature (SANCHEZ, 2011). Soil samples were obtained in a Cerrado *sensu stricto* (SS), in a riverbank

gallery forest, hereafter called "Mata de galeria" (MG), a semi-deciduous forest (FSD) and a shrubland (CD) (Figure 2).

Table 2. Physicochemical variables (mean ± SE) of the sampled sites in the PESA

Park	State Park of Serra Azul			
Type	MG	CS	FSD	SS
SWC (% H_2o g^{-1} DS)	16.7 ± 2.4	14.8 ± 1.6	17.7 ± 4.3	10.0 ± 1.2
Clay (g kg^{-1})	283 ± 8	408 ± 8	283 ± 22	283 ± 8
Sand (g kg^{-1})	600 ± 29	383 ± 22	542 ± 30	658 ± 8
Silt (g kg^{-1})	117 ± 22	208 ± 17	175 ± 25	58 ± 8
pH in H_2o	4.83 ± 0.09	4.73 ± 0.09	5.00 ± 0.17	5.07 ± 0.03
pH in KCl	3.50 ± 0.06	3.67 ± 0.03	3.90 ± 0.26	3.67 ± 0.03
P (mg dm^{-3})	4.57 ± 0.35	0.77 ± 0.09	8.90 ± 6.06	3.17 ± 0.79
Ca ($cmol_c$ dm^{-3})	0.77 ± 0.12	0.57 ± 0.09	0.70 ± 0.06	0.63 ± 0.09
Mg ($cmol_c$ dm^{-3})	0.53 ± 0.15	0.33 ± 0.09	0.47 ± 0.12	0.40 ± 0.00
K ($cmol_c$ dm^{-3})	0.10 ± 0.01	0.10 ± 0.01	0.35 ± 0.04	0.13 ± 0.01
Na ($cmol_c$ dm^{-3})	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.02	0.01 ± 0.00
CTC ($cmol_c$ dm^{-3})	6.67 ± 0.33	5.33 ± 0.33	7.00 ± 1.15	6.33 ± 0.33
Al ($cmol_c$ dm^{-3})	1.87 ± 0.23	1.50 ± 0.10	1.40 ± 0.81	1.40 ± 0.20
H+Al ($cmol_c$ dm^{-3})	5.27 ± 0.13	4.50 ± 0.10	5.60 ± 1.14	5.27 ± 0.13
C (g kg^{-1})	23.97 ± 2.40	21.83 ± 0.60	43.00 ± 9.00	24.40 ± 1.18
OM (g kg^{-1})	41.20 ± 4.13	37.20 ± 0.99	73.97 ± 15.49	41.97 ± 2.02
B (mg dm^{-3})	0.20 ± 0.04	0.21 ± 0.07	0.29 ± 0.06	0.35 ± 0.06
Cu (mg dm^{-3})	0.32 ± 0.01	0.32 ± 0.01	0.44 ± 0.05	0.42 ± 0.04
Fe (mg dm^{-3})	337.67 ± 33.89	157.67 ± 8.51	135.67 ± 34.37	288.00 ± 15.28
Mn (mg dm^{-3})	44.40 ± 18.09	4.06 ± 0.16	53.27 ± 23.20	17.90 ± 7.11
Zn (mg dm^{-3})	1.80 ± 0.32	0.98 ± 0.04	3.22 ± 0.80	1.55 ± 0.23
S (mg dm^{-3})	5.40 ± 1.39	7.33 ± 0.54	4.97 ± 1.35	6.13 ± 1.71

, SWC: Soil water content. [+]Measurement from only one sample. MG: Mata de galeria; SS: Cerrado *sensu stricto*, CS: Campo sujo, FSD: Floresta semi-decídua.

Figure 2. Photographs of the sites sample in the two parks: PNCV – Parque Nacional da Chapada dos Veadeiros; PESA – Parque Estadual da Serra Azul. (SS) Cerrado *sensu stricto*, (MG) Mata de galeria, (CL) Campo limpo, (CR), Cerrado rupestre, (FSD) Floresta semi-decídua, (CS) Campo sujo.

DNA extraction and sequencing

DNA was extracted from 0.5 g of soil with the FastDNA Spin Kit (MP Biomedicals) with additional treatment using solutions steps 2 and 3 from the PowerSoil DNA Isolation Kit (MO Bio Laboratories Inc.) to achieve maximum DNA yields with least of organic contaminants. The extraction was evaluated in 1% agarose gel electrophoresis. The average concentration of each 24 DNA samples was of 100 ng/µL (Invitrogen Qubit fluorometer dsDNA BR Kit).

Approximately 2 µg of DNA was sent to sequence on 454 platform GS FLX + technology (Macrogen, Inc., South Korea) from each sample. Two 454 plates were used to sequence, one for each park; DNA from each site constituted one-quarter of the plate. Raw sequences were uploaded to the MG-RAST server, assigned to the projects SISBIOTA_PESA_2013 (ID 6701; accession numbers 4549601.3-4549612.3) and SISBIOTA_PNCV_april_13 (ID 5456; accession numbers 4530784.3-4530795.3), and processed with default quality control pipeline.

A total of 1,364,104 sequences (average size of 746bp and 515 bp, before and after quality control in MG-RAST) for PNCV and 992,685 sequences (average size of 659 bp and 382 bp, before and after quality control in MG-RAST) for PESA. After quality control, unassembled sequences were assigned to the taxonomic annotation with BLASTX against the M5NR non redundant databases, e-value of 10^{-5}, 80% of identity cutoff and 50 bp alignment. Functional annotation was performed against the metabolic subsystems SEED database with e-value of 10^{-5}, 60% of identity cutoff and 15 bp alignment, as default. The MG-RAST table format of sequences associated with total organism abundance (best hit classification), total bacteria assignment, total subsystems, and nitrogen metabolism were downloaded and transformed to wide format to R analysis.

In addition to the analyses of N metabolism annotated genes in PNCV and PESA soils, we compared our results with other metagenomes obtained in the Cerrado biome in a study of comparison between native and managed areas: MG-RAST ID's 4577669.3 to 4577672.3, 4578924.3 to 4578927.3 and 4578714.3 (Souza *et al.*, 2016).

Statistical analysis

All analyses were conducted in R version (3.2.2). One-way ANOVA tests were used to make multiple comparisons within each park. Tukey-Kramer post-hoc tests was used when statistical difference was significant ($p<0.005$). Differences for physicochemical and

metagenomics data between parks and between SS or MG present in both parks were tested with T-test or the non-parametric Wilcoxon test. Statistical analysis with the relative abundance of either annotated taxonomy or metabolisms did not consider unclassified reads. Relative abundance is meant as the number of annotated genes for a certain classification (either of taxonomy or functional) divided by the total of annotated genes for each sample. Principal component analysis (PCA) were constructed in R with *prcomp* function set to TRUE for correlation, considering that physicochemical variables have different scales and variance. PCA were made with *FactoMineR* and *factoextra* packages. All graphs in the boxplot format were prepared in R with the *ggplot2* library as described previously (Catão *et al.*, 2016).

Results

Study sites and soils characteristics

Soil NH_4^+-N and NO_3^- -N concentration, pH and water soil content were very similar in all 8 sites sampled in the two parks (Figure 3). The content of soil water, NO_3^- -N, NH_4^+-N, and organic carbon, was measured in only one of the PNCV riverbank replicates due to the abundant presence of roots on the other replicates, which did not allow accurate measurements. The replicate of MG in PNCV had the highest NH_4^+-N and NO_3^- -N. NO_3^- -N was higher in the PESA MG than CD or SS, but no difference compared to FSD. pH was higher in the sites sampled in PNCV than PESA, but were not different within each park. However, MG sites from the two parks differed in pH. Sites sampled in PESA did not differ in soil water content, but in PNCV, CL had higher soil water content than SS and CR. CR had the soil with the least water content in the two parks. Soil in the PNCV SS was slightly moister than the PESA SS.

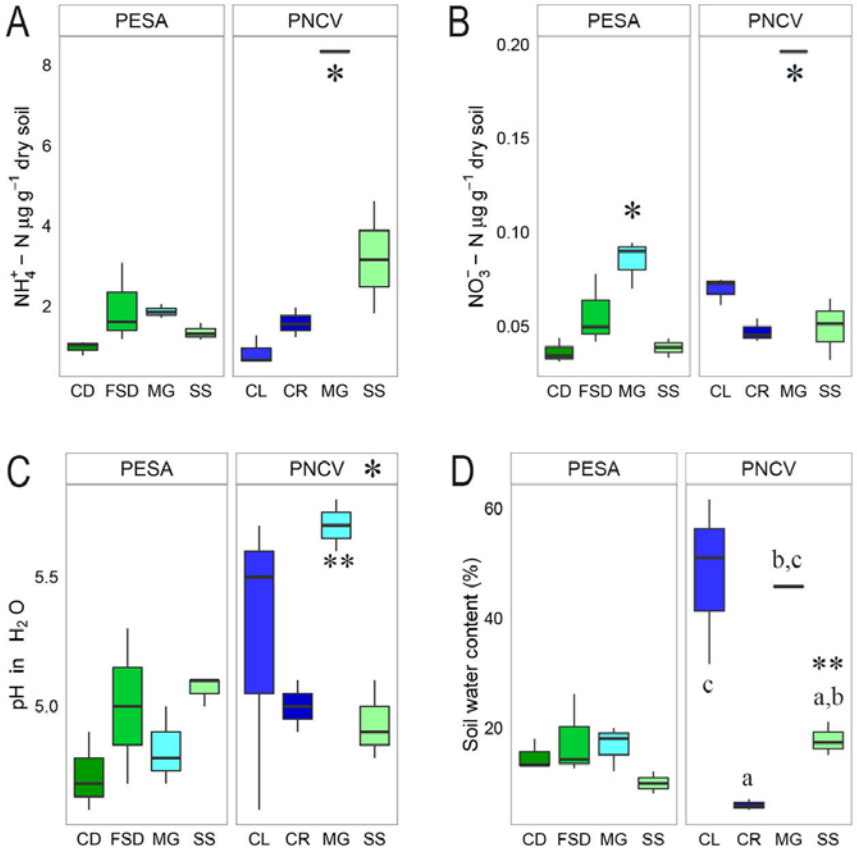


Figure 3. Boxplots on soils (A) NH_4^+-N and (B) NO_3^- -N concentration, (C) pH and (D) water soil content. One-way ANOVA or T- tests with tukey–Kramer post hoc tests to compare group means (R with the ggplot2 package) are represented with letters or with one asterisk (*) if only one site was significantly different from others. Two smaller asterisks (**)

depict statistical difference between MG or between SS present in the two parks.

On the other hand, considering several physicochemical variables in a PCA, the two parks form segregated clusters (Figure 4). PNCV is a conservation unity representative of "Altitude Cerrado", at 1200 m of altitude approximately, oppositely to PESA, that is at 650 m altitude. As altitude masked the effect of other variables, it was not considered in the PCA. Besides altitude, pH, clay (and sand), C content, Al^{+3}, cation exchange capacity, S, Fe, K and other micronutrients as B, Cu, Mn and Zn differ between the sampled vegetation, and consequently create two clusters according to the two parks.

The parks have different soil texture, PESA presenting a greater clay content then PNCV, except for the Cerrado *sensu stricto*, which had the highest clay content within the PNCV sites (Figure 4). Therefore, clay content in both SS from the two parks were not different. On the other hand, MG from PESA had higher clay content than MG in PNCV. In PESA, CD had the highest clay content.

Carbon content was similar along the sites sampled, except on the MG in PNCV. The soil in this same site had the highest cation exchange capacity (CEC), which was significantly different from the MG site in PESA (Figure 4). Similarly, SS sites differed in CEC between parks. Sulfur concentration was different between parks, especially due to S concentration in CL and MG in PNCV. Al^{+3} concentration was high in all sites sampled, but significantly higher in the PNCV MG. On the other hand, Fe concentration changed between sites within each park: SS in PNCV had significantly higher Fe content. Similarly, MG and SS had higher Fe concentration than CD and FSD in the PESA. Furthermore, MG sites from the two parks differed in Fe concentration.

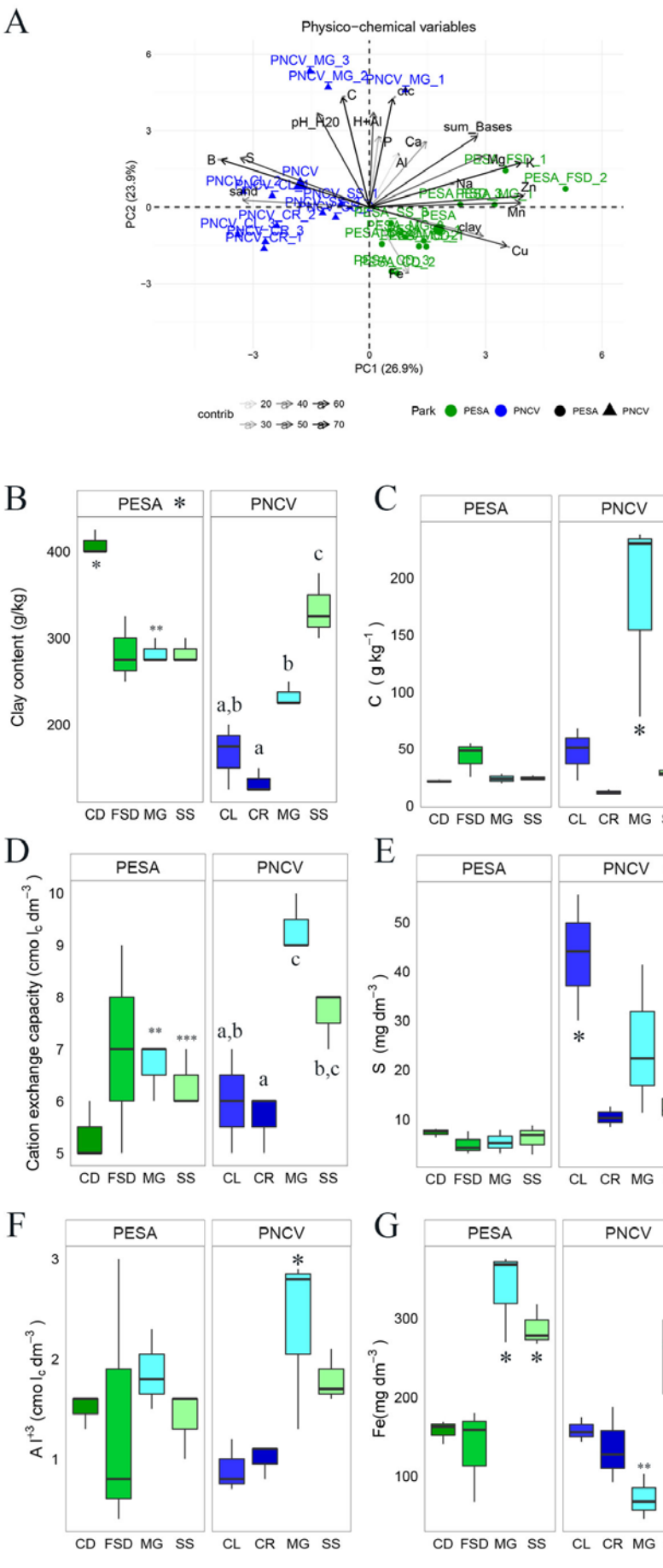

A
Physico−chemical variables
PC1 (26.9%)
PC2 (23.9%)
contrib
Park
PESA
PNCV
B
Clay content (g/kg)
C
C (g kg−1)
D
Cation exchange capacity (cmo lc dm−3)
E
S (mg dm−3)
F
Al+3 (cmo lc dm−3)
G
Fe(mg dm−3)
CD FSD MG SS
CL CR MG SS

Figure 4. Soil physicochemical variables in the two parks and their sites. (A) Principal component analysis (PCA) of soil physicochemical properties based on a correlation matrix performed with R. Each vector points in the direction in which the respective value increases. Boxplots of soils (B) clay content, (C) C content, (D) cation exchange capacity, (E) S concentration, (F) Al^{+3} concentration, (G) Fe concentration. One-way ANOVA or T- tests were performed in R. Tukey–Kramer post hoc tests to compare group means (R with the ggplot2 package) are represented with letters or with one asterisk (*) if only one site was significantly different from others. Two (or three) smaller asterisks depicted statistical difference between MG or between SS present in the two parks.

Phylogenetic and functional analyses

A total of 1,364,104 sequences were obtained from PNCV and 992,685 from PESA; an average of 7 and 11.7% did not pass on the quality control, respectively (Table S1), and 2.7 to 8.7%, respectively, were considered sequences' replicates and were excluded from the analysis. The percentage of sequences annotated to known protein was 61.4 (± 3.5) % and 46.5 (± 1.4) % for the PNCV and the PESA, respectively, and only a small fraction (around 0.5%) of the reads was annotated as ribosomal, or to the N metabolism (around 1%).

The number of ribosomal sequences annotated varied from 88 to 775 and taxonomical assignment was against the non-redundant protein M5NR database. According to this database, most of the genes annotated were from *Bacteria* (around 97%), with the remaining being part either of the Domain *Archaea* (0,9%), the Domain *Eukarya* (1,6%) or unknown (0,18%). *Archaea* was mainly present in soil as Thaumarchaeota and Crenarchaeota; CR and MG from PNCV and FSM and SS from PESA – presented low values of Euryarchaeota. The most abundant phyla in the *Bacteria* domain were Actinobacteria, Proteobacteria and Firmicutes, especially the class Bacilli, Clostridia (both from Firmicutes), α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria. Ascomycota, Basidiomycota, Streptophyta and Arthropoda were within the most *Eukarya* annotated sequences.

Contrary to the PCA constructed with the physicochemical variables, the PCA representing the phylum relative abundance shows no separation between the parks (Figure 5). The vectors point to a greater relative abundance present in some of the replicates as for example Proteobacteria and Spirochaetes for the Campo limpo site at the PNCV.

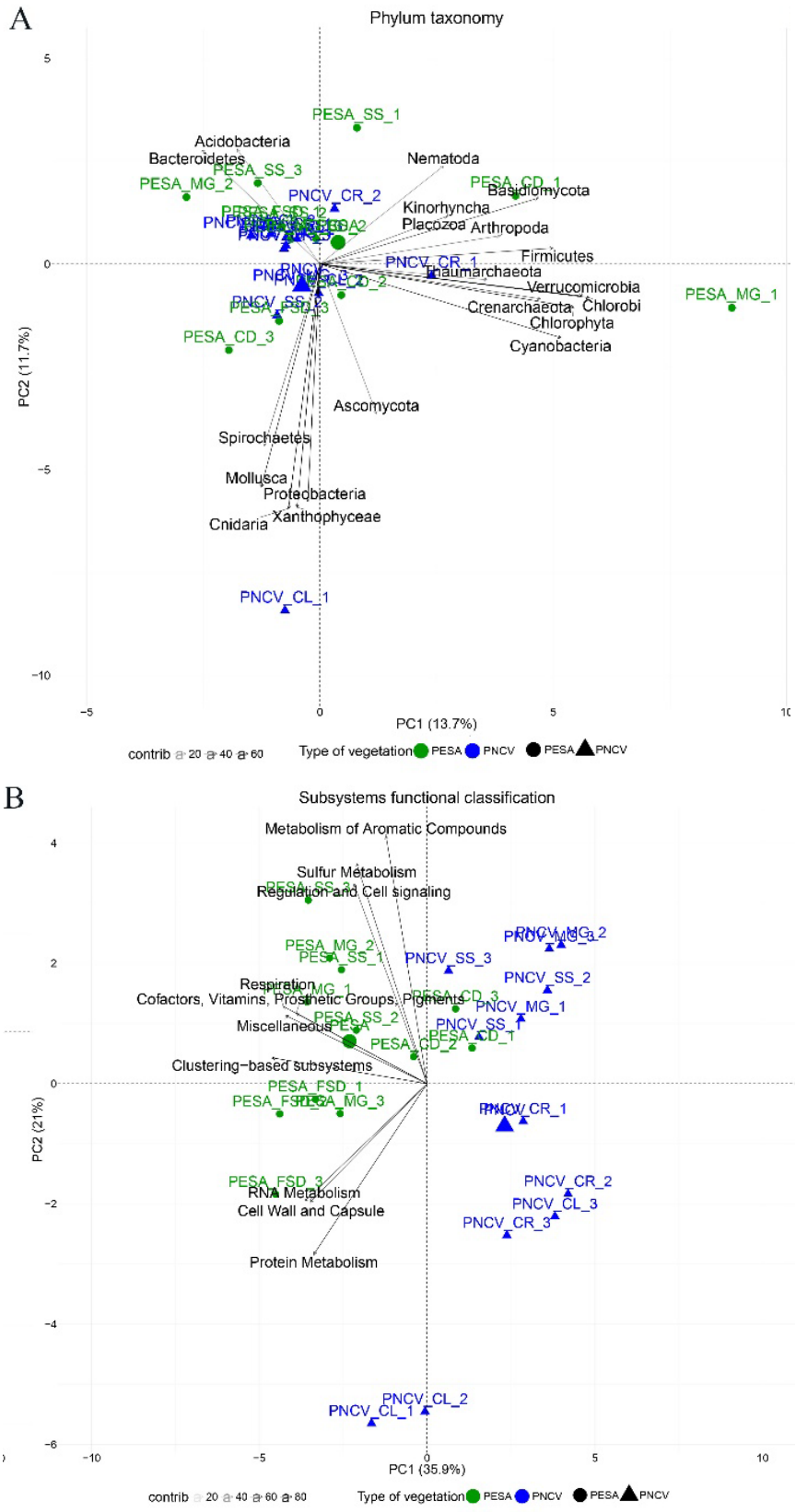


Figure 5. Principal component analysis (PCA) constructed with the relative abundance of annotated genes for (A) phylogenetic assignment of phyla and (B) subsystems functional classification based on a correlation matrix performed with R. Each vector points in the direction in which the respective value increases.

Distribution of the relative abundance of the SEED subsystems classification presents a greater separation of sites sampled in each park (Figure 5B). PESA presented significantly higher relative abundance than PNCV for most of the subsystems as seen in the PCA, but more specifically for respiration (p-value=0.014), potassium metabolism (p-value<0.0001) and phages, prophages, transposable elements and plasmids (p-value=0.044) (Figure 6). On the other hand, PNCV had more virulence, disease and defense (p-value<0.0001) annotated sequences than PESA. Despite the broad potential for metagenomes analysis, this work focused on the nitrogen metabolism.

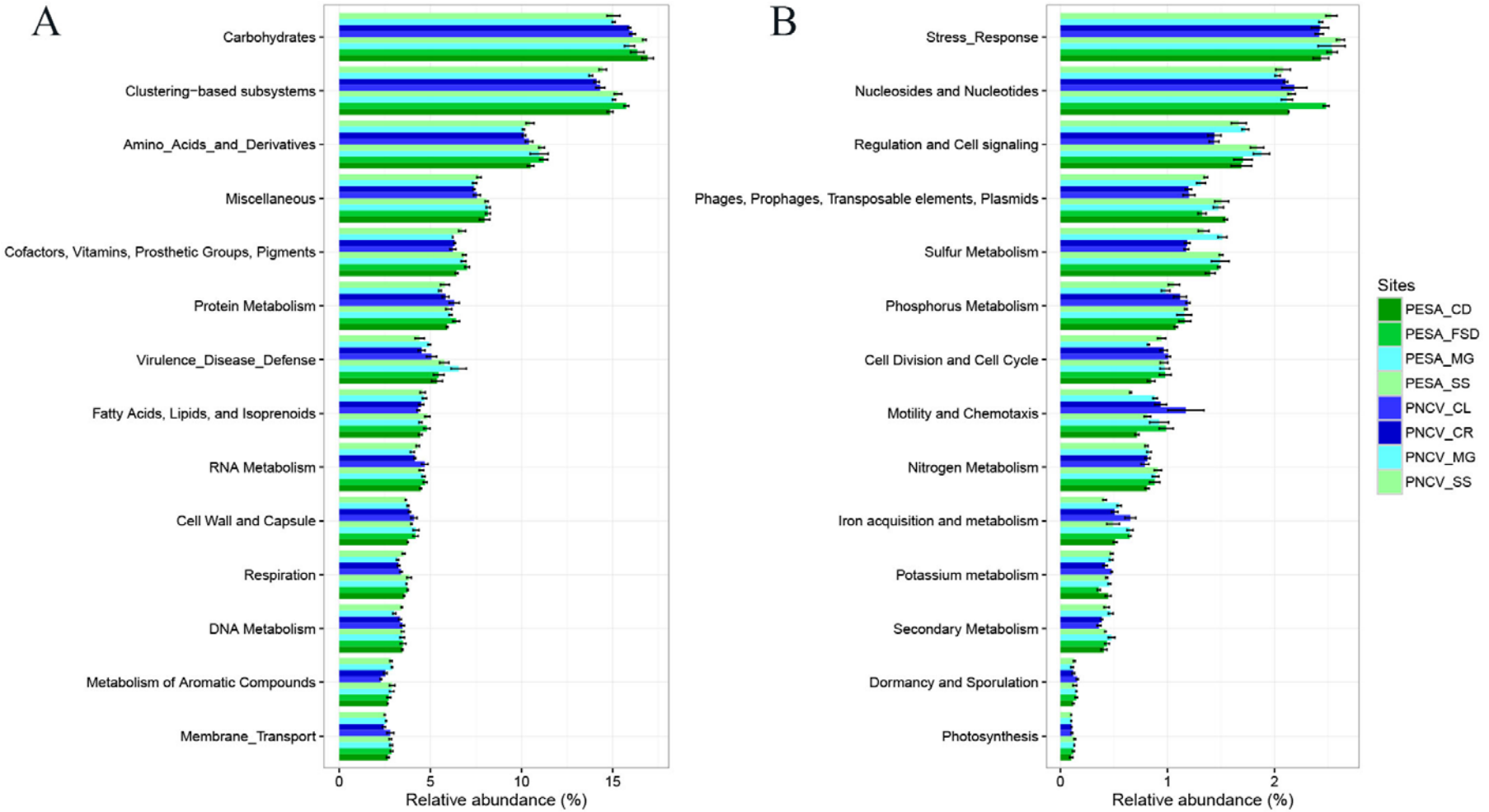


Figure 6. Bar plots for the relative abundance of SEED subsystems according to each site × park. (A) Most abundant SEED subsystems, (B) less abundant SEED subsystems.

The greatest part of annotated genes to N metabolism were related to the ammonia assimilation (37%), followed by nitrate and nitrite ammonification (17%), nitric oxide synthase (12 %) and allantoin utilization (9%) as shown in Figure 7 that concatenates all genes annotated to N metabolism in the 24 metagenomes. The arrows are proportional to the number of genes annotated in our metagenomes. The least abundant were the cyanate hydrolysis (6%), the denitrification (5%), the dissimilatory nitrite reductase (5%), the nitrogen fixation (4%), the nitrosative stress (4%) and some genes related to the amidase clustered with urea and nitrile hydratase functions (1%) and nitrilase subsystems (<1%). Only two hits were found for ammonia monooxygenase, which is an enzyme part of nitrification process, but classified in the transport system according to SEED subsystems. No nitrite oxidoreductase was detected in the metagenomes, therefore both ammonia and nitrite oxidation were represented by slim arrows.

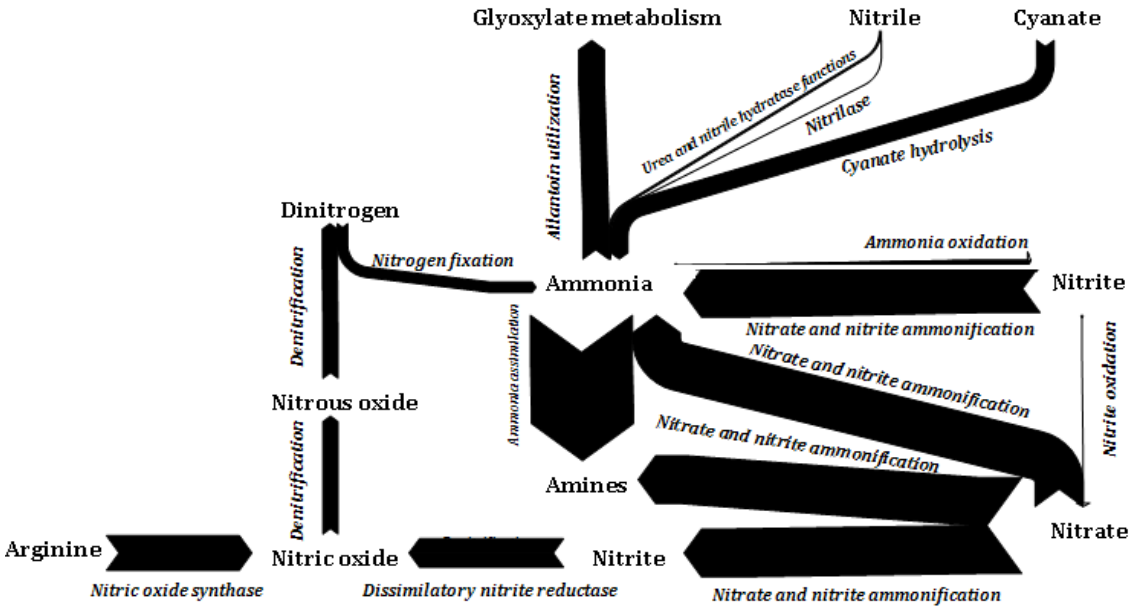


Figure 7. Schematic representation of the N cycle according to the SEED subsystems annotated genes performed with e!Sankey 2. The total number of genes annotated from PESA and PNCV metagenomes. The arrows are proportional to the number of genes annotated for each process.

PNCV and PESA were not different for the annotated genes of N metabolisms, except for the ammonia assimilation metabolism, as PNCV had significantly (p-value=0.01727) lower relative abundance than PESA (Figure 8). Ammonia assimilation was mainly represented by ammonium transporter, glutamate synthase and glutamine synthetase type I. No other N-related metabolism was different between parks. Likewise,

the Cerrado *sensu stricto* (SS) and the Mata de galeria (MG- riverbank) from both parks had similar relative abundance for the N processes displayed in Figure 8. The process of input of nitrogen to the soil system through nitrate and nitrite ammonification (genes for assimilatory nitrate reductase and nitrate/nitrite transporters) was not different between parks, but within the PNCV, the Campo limpo site was significantly lower from the Cerrado rupestre (p-value=0.0237). In contrast, Campo limpo soil had the highest relative abundance of genes annotated for nitrogen fixation (p-value=0.0249), represented by genes for nitrogenase. Similarly, Mata de galeria in PESA had higher annotated genes for denitrification than Campo sujo (p-value=0.0202), and higher annotated genes for nitrosative stress than Campo sujo and Floresta semi-decídua (p-value=0.0117). Denitrification process includes genes for nitrite, nitric oxide and nitrous oxide reductases. In all soils, the copper nitrite reductase was found, usually monitored by the nirK gene, but only in one soil from PNCV we could detect the cytochrome cd1 nitrite reductase. Annotated genes for the nitric oxide reductase quinol-dependent were significantly more numerous than other denitrification genes. Nitrosative stress, denoted by anaerobic nitric oxide reductase flavorubredoxin and hydroxylamine reductase, was also higher in the PNCV Campo limpo than Cerrado *sensu stricto* and Mata de galeria (p-value=0.00622).

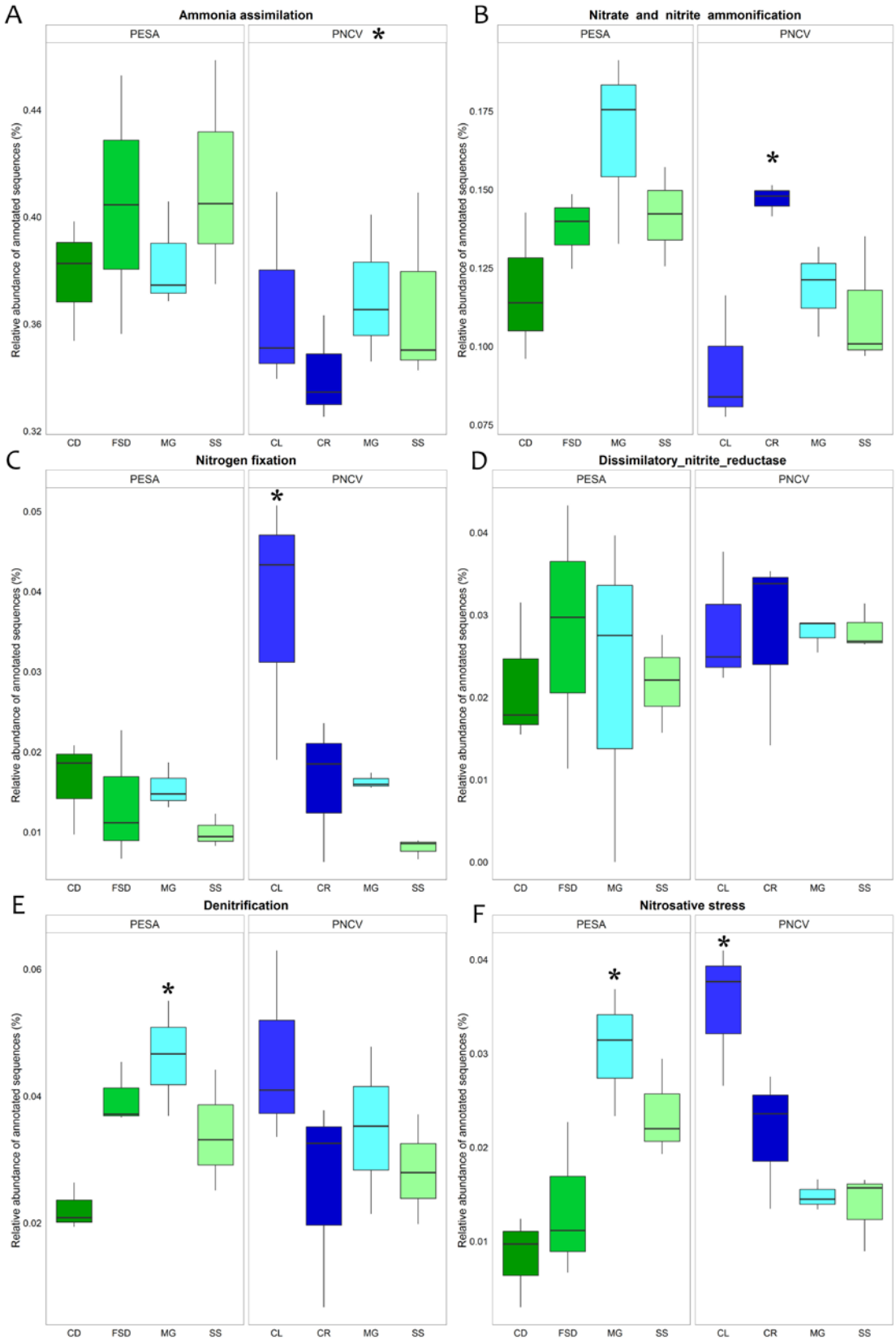


Figure 8. Boxplots of soils (A) ammonia assimilation, (B) nitrate and nitrite ammonification, (C) nitrogen fixation, (D) dissimilatory nitrite reductase, (E) denitrification, (F) nitrosative stress. One-way ANOVA or T- tests were performed in R. Tukey–Kramer post hoc tests to compare group means (R with the ggplot2 package) are represented with one asterisk (*).

Discussion

Here we present the first metagenomic description on the N cycling functional and phylogenetic genes from Cerrado soils microbiota in Central Brazil. This biome is composed by a gradient of trees/shrubs layer ranging from grasslands to forests and savannas. Analysis of phospholipid fatty acids and 16S rRNA genes have showed that vegetation cover influences the soil taxonomic microbial composition (Araujo *et al.*, 2012; Mendes *et al.*, 2012; Viana *et al.*, 2011). Nevertheless, our first hypothesis was rejected, since we could not find patterns that explained distribution of functional guilds according to the macro distribution of Cerrado's vegetation.

The types of vegetation sampled here differed in terms of soil physicochemical variables and were more similar within each park. The world literature shows pH as the factor that better explains soil microbial distribution (Lauber *et al.*, 2009). Though, for the Cerrado it has been shown that the first rains on the beginning of the rainy season or experimentally the addition of water promote greater difference on the microbial community either with increase on microbial biomass (da Silva, 2004; Nardoto and Bustamante, 2003), microbial activity and nitrification rates (da Silva, 2004), or change on the bacterial composition with the transition between the dry and rain seasons (Bresolin *et al.*, 2010; Nardoto and Bustamante, 2003; Pinto *et al.*, 2006). Furthermore, water effect on the microbial community masks the fire effect (Viana *et al.*, 2011). Truly, water promoted a change in the microbial community performing the N cycling, particularly in the Campo limpo. This confirms our second hypothesis that considers a variation in N metabolism according to soil characteristics between types of vegetation.

In fact, the soils in the PNCV had higher pH and S concentration than soils in PESA, which, in turn, presented higher clay content. However, no significant correlation was observed between pH and phylogenetic or functional genes relative abundance. Microbial community described with these metagenomes seem to differ more due to soil characteristics than the type of vegetation or the geographic distance. The similarity found between the Cerrado *sensu stricto* (SS) and the Mata de galeria (MG- riverbank) from both parks refutes the hypothesis of geographical differentiation between islands of Cerrado distant from each other in terms of soil microbial community. On the other hand, it reinforces the observed differences accordingly to type of soil and vegetation.

Assuming that types of vegetation would influence microbial distribution, the MG sites from the two parks should be similar within each other and with the FSD, as they are

forest formations. However, they should be less similar to the savanna sites - SS and CR - or the sites with predominant herbaceous layer as the CS and the CD. On the other hand, geographic distance is informative, since microbial communities are similar in the parks, and are distant 500km, therefore, there is an indication that Cerrado biome has a particular soil microbial community. This will have to be confirmed in a biogeographical model, as for example the use of mantel test with other Cerrado areas and other biomes.

As microbes are confined to a thin layer of water in the soil particles, it is reasonable to think that water is the major limitation of prokaryotic life in soil (Fenchel, 2012). The soil texture influences the water retention according to the percentage of clay, sand and silt particles, which has an impact on the gravimetric soil water content and consequently on the microbial community. The Cerrado rupestre was the driest soil sampled with the greater composition of sand in comparison to clay. This type of vegetation is only present in some fragments of the biome Cerrado specially in higher altitudes between 800 and 2000 m, characterized by rocky outcrops with high vegetal endemism and usually found in Leptosols (neossolos litólicos, Brazilian soil classification). The metagenomes found in this vegetation had the greatest α-diversity of Shannon (data not shown), potentially a greater diversity in response to the nutrient and water stress.

The theory of pore connectivity favors the idea that low contact between organisms because of low water potential allows for greater microbial diversity (Carson *et al.*, 2010). However, in the metagenomes, both the CR and the CL presented the highest Shannon diversities and they have also the two most distinct soil water content. Therefore, we considered that in this case, the higher diversity is due to disturbance (water lodging in the case of CL) promoting stochasticity for different groups to prevail instead of a higher abundance of one or another set of microorganisms.

Bacteria was the predominant domain of annotated sequences as expected because of this domain abundance in soil, the technique and the databases, and as described in the literature (Delmont *et al.*, 2012; Fierer *et al.*, 2012a). The phyla more abundant in these soils were Actinobacteria, Proteobacteria and Firmicutes. However previous work on Cerrado samples had shown by 16S rRNA pyrosequencing that the Acidobacteria was the most abundant phyla (Araujo *et al.*, 2012). These contrasting results might be because of the two different techniques used and may be an indicative of the amplicon sequencing bias as the taxonomic classification of the metagenomes here was produced by the annotation of all sequences against the protein non-redundant database M5NR. It can be also that the soil samples used in that work were different from the one

used here, since Fierer *et al.* showed a high correlation (r^2=0,81, p<0,001) of 16S rRNA and metagenomic results from soil samples of different types of biomes (Fierer *et al.*, 2012b).

The greater number of the Actinobacteria and Firmicutes found here might be associated to organic matter degradation, especially Actinobacteria which are able to degrade high C:N ratio organic matter as those found in Cerrado soils (Nardoto and Bustamante, 2003). Actinobacteria are also related to the antibiotic and secondary metabolic production (Gomes *et al.*, 2000; Petinate *et al.*, 1997) and have genes involved on ultraviolet and hydric stress (LeBlanc *et al.*, 2008) besides the apparent resistance to heavy metals (Gremion *et al.*, 2003) that might be an interesting characteristic considering the high aluminum content present on Cerrado soils.

Cerrado soils are typically N-limited, with a higher concentration of NH_4^+ than NO_3^- (Bustamante *et al.*, 2006; Nardoto and Bustamante, 2003). In addition, Nardoto and Bustamante (2003) observed during the rainy season an increase of mineralization and nitrification rates, but inorganic nitrogen concentration decreased (Nardoto and Bustamante, 2003), which could be interpreted as an assimilation of N by the vegetation. This corroborates the high percentage of ammonia assimilation genes annotated in the metagenomes (37%), that was significantly different between the parks, although these did not show a significant difference for NH_4^+-N concentration. Ammonia assimilation collected genes for the enzymes related to glutamate and glutamine synthase pathways (EC 1.4.1.13 and EC 6.3.1.2, respectively). These enzymes use one molecule of ammonia to synthesize central amines for the cell and were specially related to Bacteria, but also in *Archaea*, Cyanobacteria, Ascomycota and Streptophyta sequences.

Moreover, only two ammonia monooxygenase were retrieved in the annotated genes in the transporter membrane subsystem, which is also corroborated with the literature that suggests low levels of nitrate in Cerrado soils and correspondent low levels of nitrification rate (Bustamante *et al.*, 2006; Nardoto and Bustamante, 2003). Nitrification genes were absent in other metagenomes from Brazilian mangroves sediments (Andreote *et al.*, 2012). These AMO genes are potentially from genomes of *Methylococcus*, a methane oxidizing bacterium able to ammonia oxidation (Dalton, 1977). Although, 2 hits are too low to take conclusions from, further studies should consider *amoA* and *pmoA* comparison in Cerrado soils.

This is also validated by the low abundance of ammonia oxidizers detected with qPCR (10^{+3} to 10^{+5} *amoA* gene abundance g^{-1} soil) in undisturbed Cerrado soils (Catão *et al.*, 2016). Therefore, the low detection of genes for nitrification in the metagenomes was most

likely due to depth of sequencing. Another study with Cerrado metagenomes sequenced with Ion torrent technology (mean 2.326.852 annotated genes) obtained an average of 3 ammonia monooxygenases in native soil compared to 30 and 34 hits for no-tillage and conventional tillage, respectively (Souza *et al.*, 2015). These were from *Archaea* (Thaumarchaeota, Nitrosopumilales) and Bacteria from Alfa- (Rhodospirillales), Beta- (Burkholderiales and Nitrosomonadales) and Gamma-Proteobacteria (Pseudomonadales, Methylococcales).

In order to ammonia to be available No_3^- and No_2^- can be reduced to NH_4^+ in a process called ammonification, that corresponded to 17% of genes related to the N metabolism in the metagenomes. These were in majority nitrate transporters, nitrate (EC 1.7.99.4) and nitrite reductases (EC 1.7.1.4) from bacteria, although another work found these from plants and fungi in snowpacks (Larose *et al.*, 2013). In soil the greatest part of inorganic N is made available through mineralization from soil organic matter, and nitrification was thought to be inhibited in the acidic Cerrado soil (Catão *et al.*, 2016). Further studies should evaluate nitrate absorption, since little nitrate accumulation in soil might be a result of a rapid assimilation after nitrification.

In the Cerrado soils, 16 to 44 kg of N ha^{-1} $year^{-1}$ enter the lithosphere via biological N fixation, constituting the main form of input of N in the lithosphere, compared to N deposition (Cleveland *et al.*, 1999). N fixation is responsible for the rates of N cycling in the Cerrado ecosystems together with type of vegetation, fire frequency and land use modification (Bustamante *et al.*, 2012c). The metagenomes showed that 4% of the total of N metabolism annotated genes were of N fixation genes, and significantly higher number of genes were annotated in the *Campo limpo* than in the other vegetation types. *Bradyrhizobium* genera and other from Rhizobiales family had been already described in a Cerrado native soil (Araujo *et al.*, 2012). Moreover, the lower values of genes for ammonification in the Campo limpo might be a confirmation the input of N as N_2 to this system, as presented by a significantly higher abundance of N fixation genes. Similarly, the increase in water content in the Campo limpo was possibly related with the higher abundance of genes for nitrosative stress.

Despite the numerous species of the Fabaceae family (around 780 species) found in the Cerrado (Filgueiras, 2002), known to comprise leguminous species, the *Campo limpo* here sampled is a grassland. Furthermore, few works have measured the nodular activity in these associations (for revision see (Bustamante *et al.*, 2012c)). In fact, *Bradyrhizobium* are abundant in soils that lack leguminous plants (VanInsberghe *et al.*, 2015). These authors

described the high abundance of a *Bradyrhizobium* OTU that lacks nif and nod genes, and suggest this is a group of free-living ecotypes with potential aromatic degradation role (VanInsberghe *et al.*, 2015). Contrarily, *Campo limpo* metagenomes have nitrogenase genes, suggesting potential activity of nitrogen fixation in these soils, most likely of free-living rhizobia.

As described above, mineralization and nitrification rates are greater during the first rains on the rainy season (Nardoto and Bustamante, 2003). Recently it was proposed that climate change will impact the rain frequency and increase the length of the dry season (Bustamante *et al.*, 2012c), which might result in changes on the N cycle balance and fluxes in the Cerrado ecosystems and N trace gas emission. These are produced during nitrification and denitrification. Denitrification is a modular process responsible for the return of N_2 to the atmosphere and is favored in anaerobic environments (Graf *et al.*, 2014), but have been described in dry soils (Braker *et al.*, 2015). This trait is not centered in few clusters as the nitrification step of the nitrogen cycle, but spread within phylogenetic groups of heterotrophic organisms capable of reducing nitrate and nitrite.

Although the annotated denitrifying genes indicates that these process might be occurring in Cerrado soil particles microhotspots, N trace gases were not measured in this work. In previous studies, low N gas emissions detected in Cerrado soils were shown to be influenced by the type of vegetation and soil water gravimetric content (Pinto *et al.*, 2002). Water addition resulted in an increase of 100× on NO emissions in a Campo sujo site (Pinto *et al.*, 2002), but N_2O emissions in the Cerrado are almost always under the detection limit (Bustamante *et al.*, 2006). In the metagenomes we found all the enzymes required to denitrification: nitrite reductase (often measured by the genes *nirK*, *nirS*), nitric oxide reductase (referred to the gene *norB*), nitrous oxide reductase (measured by the presence of *nosZ* gene). The copper nitrite reductase (*nirK* gene) was significantly more abundant than its cytochrome cd1 nitrite reductase counterpart (*nirS*), as showed for other soils previously (Jones *et al.*, 2014) and in another study in the Cerrado (Souza *et al.*, 2015). In most of bacterial genomes, organisms that hold *nirK* do not possess *nirS* and vice-versa, and seems that these *nirK* and *nirS* denitrifiers respond differently to environmental gradients (Graf *et al.*, 2014). In the same way as other soils (Jones *et al.*, 2014), the abundance of nitrous-oxide reductase genes was lower than that for nitric-oxide reductase genes. Soil sink capacity for N_2O was related especially with the presence of *nosZ* denitrifiers of clade II, but the greater diversity of both clades I and II, the greater capacity of soil to reduce N_2O in excess (Jones *et al.*, 2014).

Genes for nitric oxide production from arginine were detected as 12% of genes annotated for the nitrogen metabolism. Another indicative of denitrifiers activity in Cerrado soils is the presence of nitrosative stress genes (4% of total nitrogen metabolism). This stress is promoted by high exposure of cells to nitric oxide or oxidant peroxinitrite, formed by the interaction of NO with superoxide anions. NO inhibits cell respiration and can react with multiple cell components in both prokaryotic and eukaryotic cells (Poole, 2005) and it is toxic for organisms sharing the habitat with denitrifiers (Choi *et al.*, 2006). The flooded grassland had the greatest number of enzymes related to the nitrosative stress process probably as a consequence of the anaerobic environment with higher water gravimetric content than the other Cerrado sites. On the other hand, MG from PESA had significant higher annotation of denitrification genes than other PESA sites, which was not correlated with the soil water content.

Metagenomes studies do not discuss why so often denitrification genes are annotated but fewer or none of nitrification genes are identified in the metagenomes. Some hypotheses are proposed here: 1) as denitrification is a polyphyletic characteristic, genes are widespread and have higher probability to be found, but are not necessarily active; 2) nitrification in Cerrado soils is performed by heterotrophic organisms also able to denitrify so nitrate does not accumulate (Kuenen and Robertson, 1994); 3) databases are less complete for nitrification genes than denitrification. The last assumption was tested by performing a blast of all metagenomes here sequenced against a specific *amoA* database (Pester *et al.*, 2012), and no results were found. Further studies should consider investigating heterotrophic nitrification and nitrifier-denitrification in Cerrado soils. The *amoA* gene used to monitor ammonia oxidizers targets only autotrophic organisms. One study performed in a Cerrado soil, using an inhibitor (e.g. acetylene) for the autotrophic AMO complex suggested that nitrifiers in those soils were heterotrophic (Poth *et al.*, 1995).

These results are the first set of metagenomic data representing the relative abundance of microbial genes for the N metabolism between different types of vegetation and soils from undisturbed areas in the Cerrado biome. These data will be important to understand the impact of land use change on soil microbiota on this Brazilian savanna and consequently in the ecological processes by them produced. Further investigation with these metagenomes will focus on CAZymes database, to search for specific genes related with organic matter cycling, testing again the hypothesis of difference between types of vegetation and their C and N input to the litter and soil.

Supplementary Information

Table 3. Coordinates and altitude of each sampled site

Park	Type	Replicate	Coordinates		Altitude
			S	W	(m)
PNCV	MG	1	14° 06.258'	47° 42.419'	1194
	MG	2	14° 06.246'	47° 42.428'	1170
	MG	3	14° 06.174'	47° 42.462'	1159
	CL	1	14° 06.529'	47° 42.879'	1202
	CL	2	14° 06.504'	47° 42.888'	1194
	CL	3	14° 06.480'	47° 42.883'	1196
	SS	1	14° 07.128'	47° 43.865'	1186
	SS	2	14° 07.109'	47° 43.893'	1184
	SS	3	14° 07.117'	47° 43.921'	1185
	CR	1	14° 05.499'	47° 42.265'	1187
	CR	2	14° 05.473'	47° 42.271'	1190
	CR	3	14° 05.454'	47° 42.273'	1185
PESA	MG	1	15° 50.392'	52° 14.791'	555
	MG	2	15° 50.398'	52° 14.790'	516
	MG	3	15° 50.412'	52° 14.771'	507
	CS	1	15° 49.700 '	52° 13.835'	718
	CS	2	15° 49.675'	52° 13.816'	723
	CS	3	15° 49.654'	52° 13.810'	713
	FSM	1	15° 51.118'	52° 14.854'	617
	FSM	2	15° 51.112'	52° 14.827'	621
	FSM	3	15° 51.095'	52° 14.796'	654
	SS	1	15° 50.906'	52° 14.393'	705
	SS	2	15° 50.919'	52° 14.417'	713
	SS	3	15° 50.933'	52° 14.441'	709

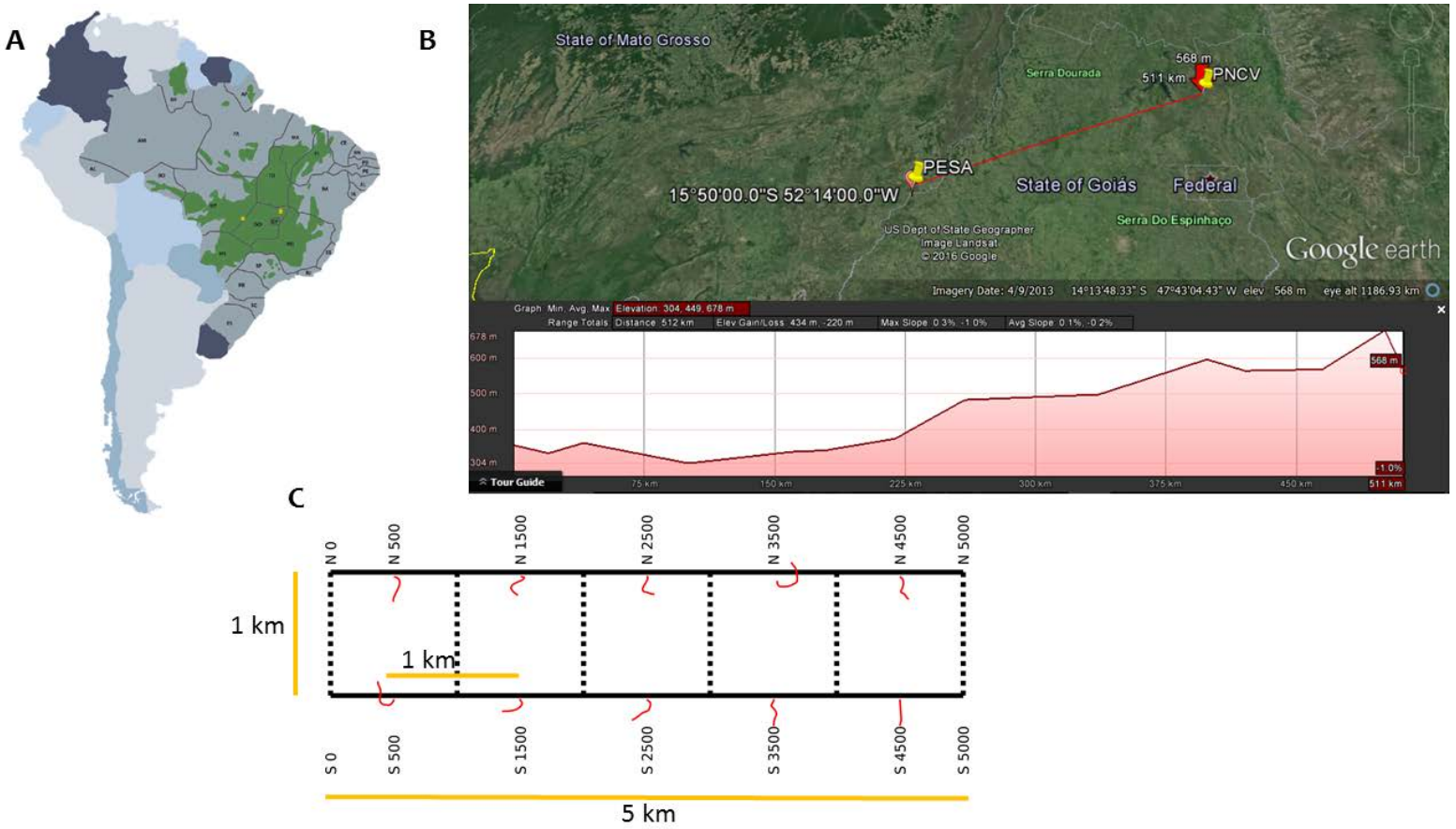


Figure 9. **(A)** Representation of the map of South America with Brazil highlighted in blue and the Cerrado biome in green. Yellow points represent the **(B)** sampled parks PESA and PNCV marked in the Google Earth photography. The elevation gradient shows a difference of 568 m and 511 km between parks. **(C)** Module experimental design scheme (figure adapted from the document of standard protocol for sampling within the Rede ComCerrado), 5 km long transects, separated by 1 km and each sampling parcel draw in red. Red lines for the parcels are not straight because they follow the local topography.

Table 4. Number of sequences for each metagenome, their identification and the number of reads that passed QC, that were annotated and to the N metabolism

MG-Rast	Site ID	Vegetation type	raw reads	after QC	%	mean length	annotated seqs	% after QC	annotated in N metabolism subsystems	% of annotated
4530784.3	N500_1		70230	62300	88.7	520+-228	42037	67.5	355	0.8
4530785.3	N500_2	Cerrado rupestre	112118	99061	88.4	499+-227	58910	59.5	467	0.8
4530786.3	N500_3		130778	115537	88.3	505+-227	79126	68.5	657	0.8
4530787.3	S1500_1		155186	137419	88.6	520+-227	95724	69.7	824	0.9
4530788.3	S1500_2	Mata de galeria	124049	109897	88.6	506+-228	74067	67.4	590	0.8
4530789.3	S1500_3		111995	99193	88.6	497+-228	68485	69.0	616	0.9
4530790.3	S2500_1		93793	87153	92.9	533+-235	60647	69.6	553	0.9
4530791.3	S2500_2		110995	102792	92.6	516+-235	67936	66.1	540	0.8
4530792.3	S2500_3	Campo limpo	117700	109072	92.7	518+-233	71091	65.2	541	0.8
4530793.3	S4500_1		134824	125346	93.0	530+-232	89215	71.2	775	0.9
4530794.3	S4500_2	Cerrado *sensu stricto*	109725	101547	92.5	528+-233	69763	68.7	611	0.9
4530795.3	S4500_3		92711	85968	92.7	516+-230	60202	70.0	468	0.8
4549601.3	3-1		29,300	26,477	90.4	369 ± 197	13490	50.9	125	0.9
4549602.3	3-2	Mata de galeria	97323	87,225	89.6	367 ± 198	42619	48.9	427	1.0
4549603.3	3-3		158,046	143,133	90.6	405 ± 206	75947	53.1	741	1.0
4549604.3	5-1		90,820	81,438	89.7	363 ± 195	40957	50.3	361	0.9
4549605.3	5-2		70,945	64,366	90.7	394 ± 204	33387	51.9	295	0.9
4549606.3	5-3	Campo sujo	138,667	124,865	90.0	382 ± 202	64187	51.4	548	0.9
4549607.3	6-1		61,706	55,778	90.4	401 ± 206	29867	53.5	258	0.9
4549608.3	6-2	Floresta semi-decídua	58,538	52,355	89.4	377 ± 200	26786	51.2	285	1.1
4549609.3	6-3		55,249	49,503	89.6	390 ± 202	26254	53.0	254	1.0
4549610.3	7-1		68,945	61,970	89.9	384 ± 204	31671	51.1	281	0.9
4549611.3	7-2	Cerrado *sensu stricto*	84,655	76,224	90.0	392 ± 205	40552	53.2	418	1.0
4549612.3	7-3		78,491	70,321	89.6	371 ± 198	36068	51.3	352	1.0

Chapter 3 - Short-term impact of soybean management on ammonia oxidizers in a Brazilian savanna under restoration as revealed by coupling different techniques [3]

"Nem tudo o que é torto é errado: veja as pernas do Garrincha, veja as árvores do Cerrado"

Nicolas Behr

Abstract

Interactions between soil characteristics and soil microbiota influence soil ecosystem processes such as nitrification however, their complexity makes interpretation difficult. Furthermore, the impact of soil management systems on abundance and activity of soil microbial community is poorly understood, especially in the Neotropics. To investigate these interactions, the effects of tillage, inorganic fertilization, and plant cover on the abundance of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were assessed by quantification of the marker gene (*amo*A) during different stages of soybean cultivation in a site under restoration from gravel extraction in the Central Brazilian Savanna (Cerrado). Results of molecular analysis and classic and isotope techniques showed that levels of organic C and NH_4^+-N were higher in the soybean field during fallow than in an adjacent undisturbed field (Campo sujo). Ammonia oxidizer abundance and nitrification rates were also higher in the agricultural soil than in the undisturbed site, with the lowest ammonium/nitrate ratio in tilled soil. Soil $\delta^{15}N$ was lower in the undisturbed soil than the agricultural soil. Both AOA and AOB were more abundant during soybean crop transitional stages, and this increase positively correlated with soil pH, particularly for AOB abundance, in tilled soil and within the soybean rhizosphere. The results suggest that AOB have more copiotrophic characteristics than AOA and are better able to change available ammonium in the soil. The combination of standard soil ecological methods and modern molecular analysis show the short-term modification of ammonia oxidizer abundance and soil N dynamics in a managed system within the Cerrado biome.

[3]Catão, E. C. P.; Lopes, F. A. C.; Rubini, M. R.; Nardoto, G. B.; Prosser, J. I.; Krüger, R. H. (2016) Short-term impact of soybean management on ammonia oxidizers in a Brazilian savanna under restoration as revealed by coupling different techniques. *Biology and Fertility of Soils*, 1-12. DOI 10.1007/s00374-015-1086-0

Introduction

The impact of land use on the functioning of soil microbiota has consequences for the processes governed by these organisms and consequently for the terrestrial ecological services that they provide (e.g., decomposition and nutrient cycling). Agriculture and managed pasture for cattle breeding have converted approximately 53% (117,870 km^2) of the Cerrado biome landscape in the last two decades (Beuchle *et al.*, 2015), with increasing alterations in floristic composition and edaphic characteristics due to fertilization, liming, and crop monoculture itself. Changes in soil use and management likely modify the C and N dynamics in these areas, leading to changes in soil C and N stocks and increases in greenhouse gas emissions to the atmosphere (Carvalho *et al.*, 2009).

Soil management and monoculture crops are associated with a decrease in total and microbial N, particularly in conventional tillage systems (Hernández-Hernández and López-Hernández, 2002). In contrast, no-till management is associated with better soil quality and higher enzyme activity (Peixoto *et al.*, 2010) and microbial C biomass (Vinhal-Freitas *et al.*, 2012). In addition, no-till farming appears to have fewer effects on the composition of microbial communities (Rachid *et al.*, 2013). Previous research has shown that the soybean plant influences the composition of the soil microbial community, with lower microbial diversity observed during plant development in soils under soybean cultivation (Bresolin *et al.*, 2010).

In the Amazonian forest, land use change alters functional gene diversity and the composition and abundance of soil microbial communities, with differences in soil pH and organic matter content linked to differences in the composition of genes, including those associated with C and N cycles (Paula *et al.*, 2014). For example, 15% to 30% of genes related to the N cycle have their abundances affected by the cultivation of bioenergy crops (*Zea mays* and *Miscanthus giganteus*) (Mao *et al.*, 2011), indicating that agriculture has an impact not only on microbial taxonomic composition but also on its potential ecological functions.

In view of the economic and ecological costs of fertilization and N losses, it is important to investigate nitrifiers in Cerrado soils to develop better soil management practices. Undisturbed Cerrado soils under native vegetation have low pH and a high NH_4^+-N:NO_3^--N ratio but very low nitrification rates (Nardoto and Bustamante, 2003) and insignificant N_2O emissions (Cruvinel *et al.*, 2011; Pinto *et al.*, 2006; Pinto *et al.*, 2002). These characteristics are often associated with a greater abundance of ammonia-oxidizing archaea (AOA) (Gubry-Rangin *et al.*, 2011; Gubry-Rangin *et al.*, 2010; Nicol *et al.*, 2008), which

appear to prefer ammonia generated from the mineralization of organic N and are the predominant ammonia oxidizers in acid soils (Levičnik-Höfferle *et al.*, 2012; Prosser and Nicol, 2012; Zhang *et al.*, 2012). In contrast, ammonia-oxidizing bacteria (AOB) are more commonly associated with nitrification in soils with higher ammonia input (Jia and Conrad, 2009); therefore, the addition of inorganic or organic N fertilizers may influence the relative abundance of AOA and AOB. The abundance of ammonia oxidizers, which perform the rate-limiting step of nitrification, can be estimated by amplification of the *amoA* gene, which encodes subunit A of ammonia monooxygenase.

Investigation of nitrification in the Cerrado biome is of particular interest because this ecosystem is N-limited (Bustamante *et al.*, 2012b) , with low nitrate content (Bustamante *et al.*, 2006; Nardoto and Bustamante, 2003) and low rates of nitrification (Bustamante *et al.*, 2006; Nardoto and Bustamante, 2003). These characteristics are usually associated with a high litter level and soil C:N ratio, leading to low availability of N and a higher rate of N immobilization than mineralization (Bustamante *et al.*, 2006; Nardoto and Bustamante, 2003).

Long-term land use is believed to modify the composition of soil microbial communities (Jangid *et al.*, 2011; Paula *et al.*, 2014), but few studies have described the short-term impacts (Lazcano *et al.*, 2013). This study investigated the short-term effects of land use change, over 134 days, on ammonia oxidizers and tested the following hypotheses: (1) AOA are more abundant than AOB in undisturbed Campo sujo soil and in soybean site during the fallow period because of lower pH and provision of ammonium mainly by net N mineralization; (2) the relative abundance of AOB is greater in agricultural fertilized soil; and (3) the relative abundance of AOB increases during crop establishment due to the increase in pH and addition of inorganic fertilizers, which are associated with an increase in nitrate content and nitrification. To test these hypotheses, changes in archaeal and bacterial *amoA* gene abundance were determined by qPCR analysis in a soybean field and in soil from an adjacent undisturbed site (Campo sujo). This work describes short-term changes in the abundance of ammonia oxidizers in soil being restored after decades of gravel extraction in the Cerrado biome by evaluating the impact of soil management on microbial communities.

Materials and methods

Study sites and soil characteristics

The field sites are located in the Cerrado biome within a commercial farm, Fazenda Tabapuã dos Pireneus, in the municipality of Cocalzinho de Goiás (Federal State of Goiás, Brazil). Average precipitation and temperature during sampling (134 days between the first and last days of sampling, October 13, 2012 and March 24, 2013, respectively), measured at the nearest meteorological center (approximately 30 km from the farm; Pirenopolis, GO, Station 83376, 15°50'60"S 48°57'36"W), were 270 mm per month (Figure 9) and 24.8°C (range 19°C–32.5°C). The climate in the Cerrado biome is tropical (Köppen Aw), and all soil samples were collected during the wet season (October to April), when 90% of the annual precipitation occurs.

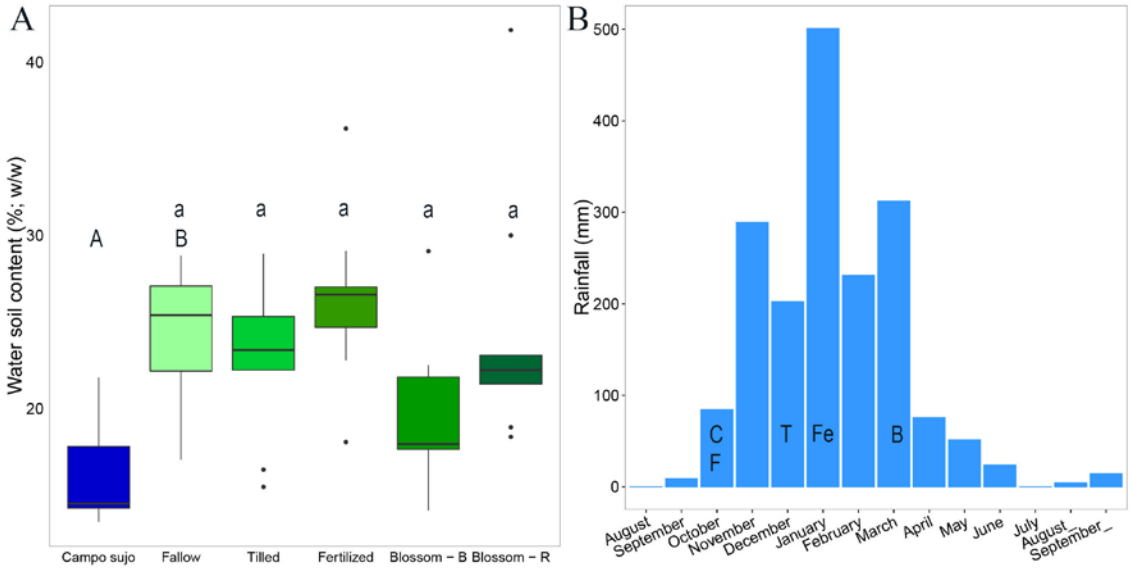


Figure 10. Gravimetric soil water content. Boxplot created by R version 3.0.2 with the ggplot2 library. Letters and corresponding colors correspond to significant differences among groups after the Tukey–Kramer post hoc test. In (B) letters represent when soils were sampled, *C* Campo sujo, *F* Fallow, *T* Tilled, *Fe* Fertilization, *B* Blossom.

This study focused on two sites: an undisturbed site dominated by grass and dispersed shrubs, known as Campo sujo (Ribeiro 2008) (15°46'01"S, 48°48'57"W) and an adjacent site (approximately 200 m away) converted to soybean crop (15°46'06"S, 48°48'55"W) (hereafter called the "soybean site"). Both sites have the same average altitude (1,118 m), rainfall, and air temperature. The soybean site, which was degraded because of gravel removal activity that occurred over decades, is in the process of restoration to become an integrated livestock-forest system. It was first cultivated in 2012,

with the establishment of maize followed by natural fallow. For maize cultivation a solution of 100 kg ha^{-1} of NPK (8:30:16) and 200 kg ha^{-1} urea were applied to the soil after plowing. Soybean seeds were then sowed after a 1-year fallow period. For soybean cultivation, an NPK mixture (8:30:16) and 8% micronutrient mixture (FPE BR12) were added to the soil at 5 cm depth. The transgenic soybean *Glycine max* Bayer variety 810 was sowed (after inoculation with rhizobia) every 10 cm in rows separated by 50 cm. Soil from the soybean site was sampled four times: after 9 months of natural fallow since the last maize cultivation (F; mid-October 2013); the day after the soil was tilled to a depth of 20 cm (T; first week of December 2012); 1 month after fertilization (FE, first week of January 2013); and at the blossom soybean stage of development (end of February 2013), at which time bulk soil (B) and rhizosphere soil (soil in direct contact with the root) (Rz) were sampled. To obtain soil from the rhizosphere, plants near the bulk soil sampling location were removed, the soil loosely surrounding the plant was released, and adherent soil at the rhizosphere was collected mechanically in a plastic bag. Figure 10 illustrates the treatments and the two study sites. Although crops in this farm are usually cultivated using no-till management, the history of gravel extraction in the soybean site necessitated use of a plow in deeper soil (20 cm). The farmer did not initially consider plowing, and only the top 10 cm (more active layer) was sampled.

Soil was obtained at nine locations at the two adjacent sites. The nine replicates were used for N concentration, pH, and soil water content measurements. However, for the remaining physicochemical data, molecular, and $\delta^{15}N$ analysis, the samples were combined into triplicate samples, according to the column numbers presented in Figure 10. In the soybean site, samples were taken from the rows. At each location, 10 soil core samples (10 cm deep, 5 cm diameter) (Figure 10) were obtained, passed through a 2-mm mesh sieve, combined, and then stored at –20°C for subsequent physicochemical and molecular analyses. Inorganic N was extracted by agitating the soil sample for 1 h in 1 M KCl (1:5 soil/solution ratio). NH_4^+-N was determined using the Nessler colorimetric method (Embrapa 1999) with a spectrophotometer set at 425 nm. NO_3^--N was determined by spectrophotometry (Mulvaney 1996) at 218 nm, subtracting interference caused by organic matter at 254 and 280 nm (Meier 1991). These measurements were considered time zero and compared with NH_4^+-N and NO_3^--N measurements after samples were incubated in the laboratory in separate closed plastic bags for 7 days at room temperature in the dark (Piccolo et al. 1994). Net N mineralization and nitrification rates were expressed as changes

in NH_4^+-N + NO_3^--N or NO_3^--N, respectively, during the 7 days of incubation. All results are expressed in per g oven-dried (105°C) soil.

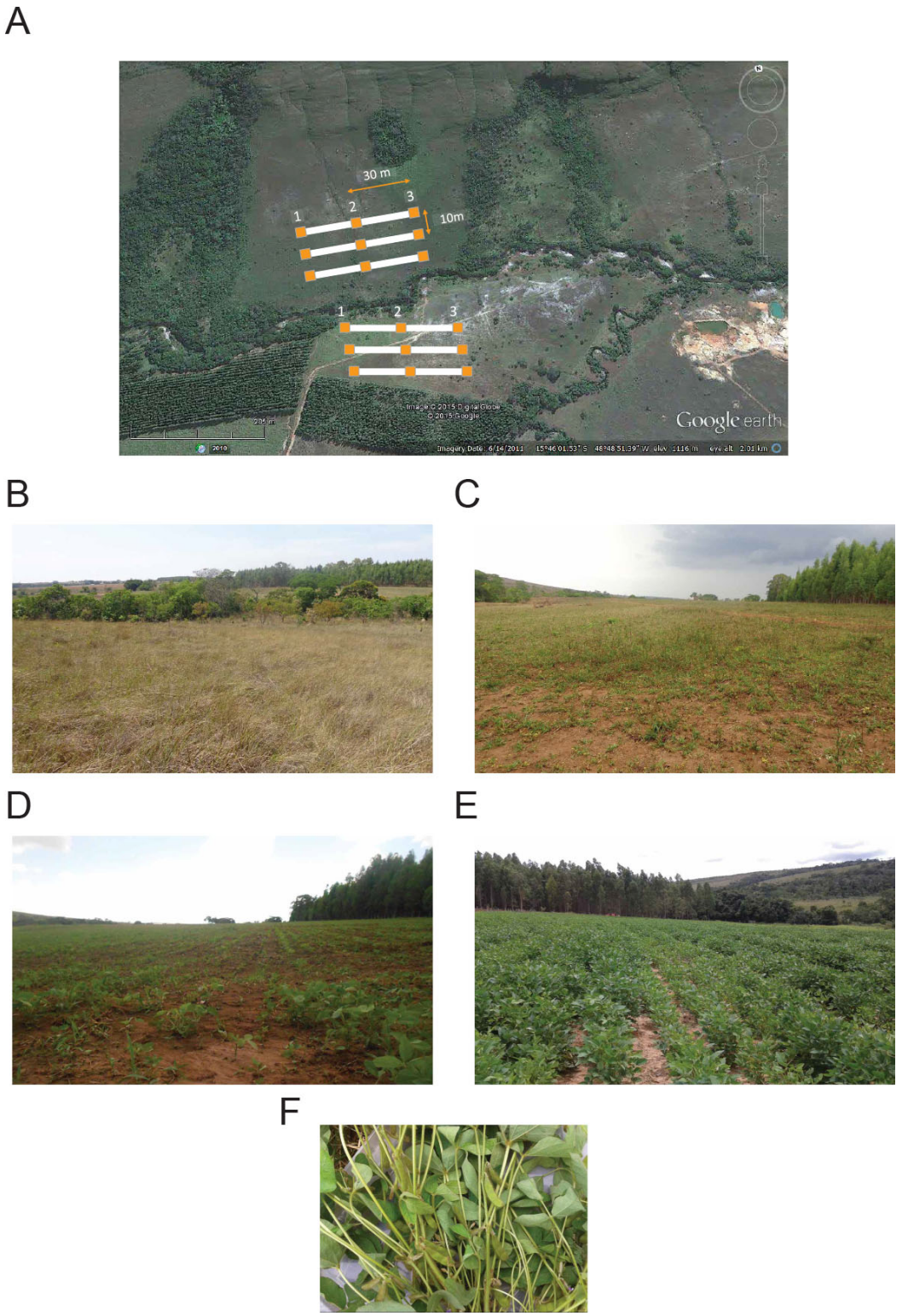


Figure 11. Satellite view and photographs of the sample site on the Tabapuã dos Pireneus Farm. (A) Schematic representation of the sampling design on a Google Earth picture from the sample site. 1–3 represent composite samples for molecular analysis. (B)–(F) Photos of

the soil collection sites. (B) Undisturbed Campo sujo site, (C)–(F) Soybean site at four different time points: (C) after 9 months of natural fallow, (D) 1 month after fertilization, (E) during the blossom stage of soybean development, (F) soybean plants with beans.

Physicochemical and molecular analyses were performed in biological triplicates. Soil texture and concentrations of macro- and micronutrients were determined by using standard methods (Soils Embrapa–SNLCS) at SoloQuímica, Inc, Brasília, Brazil. Both soils are well-aerated and well-drained. The undisturbed Campo sujo soil is classified as sandy loam with 20.8% clay, and the soybean site is a sandy clay soil with 31.7% clay. Both soils are considered to have a medium clay texture (Embrapa 2006) (Table 1). This work is not meant to compare the sites but to describe the rapid change in ammonia oxidizer abundance during the establishment of a soybean crop. The undisturbed site was used as a control to represent nitrification in a pristine Cerrado area.

Table 5. Soil physicochemical properties for each one of the replicates in all treatments

	Campo sujo		Fallow		Tilled		Fertilized		Bulk - Blossom	
SWC (% H_2O g^{-1} DS)	16.1	±0.9	21.7	±2.063	22.9	±1.873	26.3	±1.583	19.8	±1.675
Clay (g kg^{-1})	208.3	±8.3	308.3	±8.333	325.0	±14.434	333.3	±8.333	300.0	±14.434
Sand (g kg^{-1})	733.3	±8.3	600.0	±14.434	541.7	±8.333	550.0	±14.434	558.3	±16.667
Silt (g kg^{-1})	58.3	±8.3	91.7	±8.333	133.3	±16.667	116.7	±8.333	141.7	±8.333
pH (in H_2O)	5.4	±0.1	5.5	±0.058	6.0	±0.033	6.0	±0.033	6.0	±0.058
pH (in KCl)	3.6	±0.1	4.3	±0.100	5.2	±0.033	5.2	±0.058	5.0	±0.058
CEC (cmolc dm^{-3})	6.0	±0.6	6.0	±0.577	6.3	±0.333	6.7	±0.333	6.7	±0.333
Al (cmolc dm^{-3})	1.2	±0.1	0.1	±0.033	0.0	±0.000	0.0	±0.000	0.0	±0.000
N (%)	0.11	±0.00	0.12	±0.01	0.12	±0.01	0.12	±0.00	0.10	±0.00
$\delta^{15}N$	5.64	±0.08	7.05	±0.12	7.15	±0.16	7.16	±0.10	7.57	±0.14
C (%)	1.76	±0.03	2.04	±0.16	1.99	±0.12	1.92	±0.10	1.63	±0.06
OM (g kg^{-1})	42.6	±2.4	45.0	±4.159	39.1	±1.258	38.1	±2.118	36.5	±2.586
P (mg dm^{-3})	1.8	±0.1	1.2	±0.418	14.6	±6.053	14.1	±1.510	20.9	±11.767
Ca (cmolc dm^{-3})	0.4	±0.06	0.7	±0.115	2.7	±0.067	2.7	±0.338	2.7	±0.088
Mg (cmolc dm^{-3})	0.1	±0.03	0.6	±0.145	0.8	±0.033	0.7	±0.120	0.8	±0.033
B (mg dm^{-3})	0.24	±0.04	0.10	±0.039	0.46	±0.012	0.49	±0.040	0.48	±0.026
Cu (mg dm^{-3})	1.72	±0.04	1.57	±0.113	0.06	±0.020	0.05	±0.012	0.05	±0.028
Fe (mg dm^{-3})	165.40	±41.01	86.03	±6.731	106.40	±4.277	141.00	±7.000	92.37	±29.453
Mn (mg dm^{-3})	68.74	±58.82	9.01	±2.865	7.70	±0.141	7.43	±1.017	8.64	±0.380
Zn (mg dm^{-3})	1.75	±1.71	0.22	±0.101	1.65	±0.405	2.34	±0.418	3.54	±1.033
S (mg dm^{-3})	6.03	±0.15	3.20	±0.100	3.13	±0.145	4.13	±0.865	4.63	±0.835

AT: average temperature; SWC: Soil water content; CEC: cation exchange capacity; DS: dry soil; OM: organic matter.

Isotope analysis

All soil samples were air-dried and ground to a fine powder. A sub-sample of 15 to 20 mg was sealed in a tin capsule and loaded into a ThermoQuest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA, USA) coupled with an elemental analyzer (Carlo Erba model 1110; Milan, Italy). These analyses were performed at Centro de Energia Nuclear na Agricultura (CENA - USP) in Piracicaba, Brazil. The natural abundance of stable isotopes of C and N were measured in relation to recognized international standards. As standard laboratory procedure, internal working standards (atropine and soil standard no. 502-308 from LECO Corporation) were included in every run. Relative stable isotope values are reported in "delta" notation, as δ values in parts per thousand (‰) according to the molar ratio (R) of the rare to abundant isotope ($^{15}N/^{14}N$; $^{13}C/^{12}C$), i.e. $\delta‰ = (R_{sample} / R_{standard} - 1) \times 1{,}000$. The precision of measurements was ±0.3 and 0.5‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

DNA extraction

DNA was extracted from 0.5 g soil using the FastDNA Spin Kit (MP Biomedicals) with additional treatment using solutions 2 and 3 from the PowerSoil DNA Isolation Kit (MO Bio Laboratories Inc.) to achieve maximum DNA yields with the least organic contamination. The DNA was analyzed by 1% (w/v) agarose gel electrophoresis. The average concentration of each 18 DNA sample was 100 ng μL^{-1} (Invitrogen Qubit fluorometer dsDNA BR Kit).

Real-time PCR

Thaumarchaeota 16S rRNA and archaeal and bacterial *amoA* genes were amplified in an Eppendorf Mastercycler and quantified using standard curves. Each 20-µl reaction contained 1X QuantiFast master mix (for AOA) or QuantiTect master mix (for AOB) (Qiagen), 0.4 µM primers (archaeal 16S rRNA, AOA *amoA*) or 0.6 µM primers (AOB *amoA*), 2 µg μl^{-1} bovine serum albumin (Promega), and 5 ng DNA. The thaumarchaeal 16S rRNA gene was amplified with the 771f and 958r primers (Ochsenreiter et al. 2003), the AOA *amoA* gene with the crenamo23f and crenamo616r primers (Tourna 2008), and the AOB *amoA* gene with the amoA1F and amoA2R primers (Rotthauwe et al. 1997). Cycling conditions were as follows: 15 min at 95°C followed by 40 cycles of 15 s at 94°C and 1 min 30 s at 60°C for the AOA *amoA* gene; and 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for the AOB *amoA* gene. Fluorescence was measured after 5 s at

80°C (AOA *amoA*) or 8 s at 83°C (AOB *amoA*) to exclude fluorescence contamination of potential primer-dimers. Melting curves between 65°C and 95°C were analyzed for each run.

Standards were made from 10-fold dilutions of the fragment of the gene of interest. This fragment was obtained by amplification of the genes with the respective primers from a composite of the soil samples used in this work. The fragment was cloned into a pGEM®-T Easy Vector (Promega) and re-amplified using M13 primers that recognize sites flanking the cloned fragment. Three clones of each gene were selected and verified by Sanger sequencing. The longer and more accurate sequence was chosen as the standard. Plasmid DNA concentrations were verified using a Qubit 2.0 fluorometer (Life Technologies) and NanoDrop 1000 spectrophotometer (Thermo Scientific). To verify the correct size of individual PCR products, melting curve and agarose gel electrophoresis analyses were performed. To exclude the fluorescence from potential primer-dimers, fluorescence was captured after each amplification cycle above 80°C. Efficiency of amplification and r^2 values were 0.86 and 0.990 for archaeal 16S rRNA, 0.92 and 0.995 for archaeal *amoA*, and 0.86 and 0.994 for bacterial *amoA*, respectively. No inhibition was detected in assays consisting of soil DNA diluted in water or with a known amount of standard DNA.

Statistical Analysis

Statistical analyses were performed in R (v 3.0.2), and all qPCR and physicochemical data were analyzed for normality and homoscedasticity with both Kolmogorov–Smirnov and Levene's test statistics. Data that did not follow a normal distribution were log-transformed. One-way ANOVA tests were used to make multiple comparisons, with Tukey–Kramer *post hoc* tests to compare the group means shown in the graphs with different letters and corresponding colors. All graphs in the boxplot format were prepared in R with the *ggplot2* library, in which the default is to present the upper and lower sides of the box as the first and third quartile, whiskers corresponding to the highest and lowest values within 1.5 interquartile range (IQR), and dots representing outliers outside the IQR. The Pearson correlation was used to evaluate relationships between qPCR data and physicochemical variables with relevant biological implications (i.e., pH, net nitrification rate, $\delta^{15}N$). The Bonferroni (Rice 1989) or Benjamini–Hochberg (BH) (Benjamini and Hochberg 1995) methods were used to correct *p* values for multiple comparisons; the Bonferroni correction is more conservative.

Results

Description of study sites and soil physicochemical characteristics

Water content of the undisturbed soil was lower than that of the soybean site at all time points, including soil collected on the same day in the soybean site during fallow. This finding may reflect differences in soil texture (Figure 9). Fallow soil from the soybean site contained residual material from the previous maize cultivation. Before sowing, 2 ton ha^{-1} limestone was applied to the soil, which increased soil pH in H_2O from 5.5 (4.3 in KCl) to 6 (5.2 in KCl). The undisturbed Campo sujo soil had lower pH values (5.4 in H_2O and 3.6 in KCl) (Table 1).

Principal component analysis of soil physicochemical data (Figure 1) indicated that the physicochemical characteristics in the fallow soil differed significantly from soil collected in the soybean site at the other time points (Figure 11A). The undisturbed soil also differed from the fallow soil from the soybean site, which had higher organic C and NH_4^+-N concentrations (Figure 11B). However, other soils obtained from the soybean site clustered together, indicating similar physicochemical characteristics. In particular, these soils had higher pH and levels of nitrate, water, and micronutrients compared to the undisturbed Campo sujo soil and fallow soil (Figure 11B).

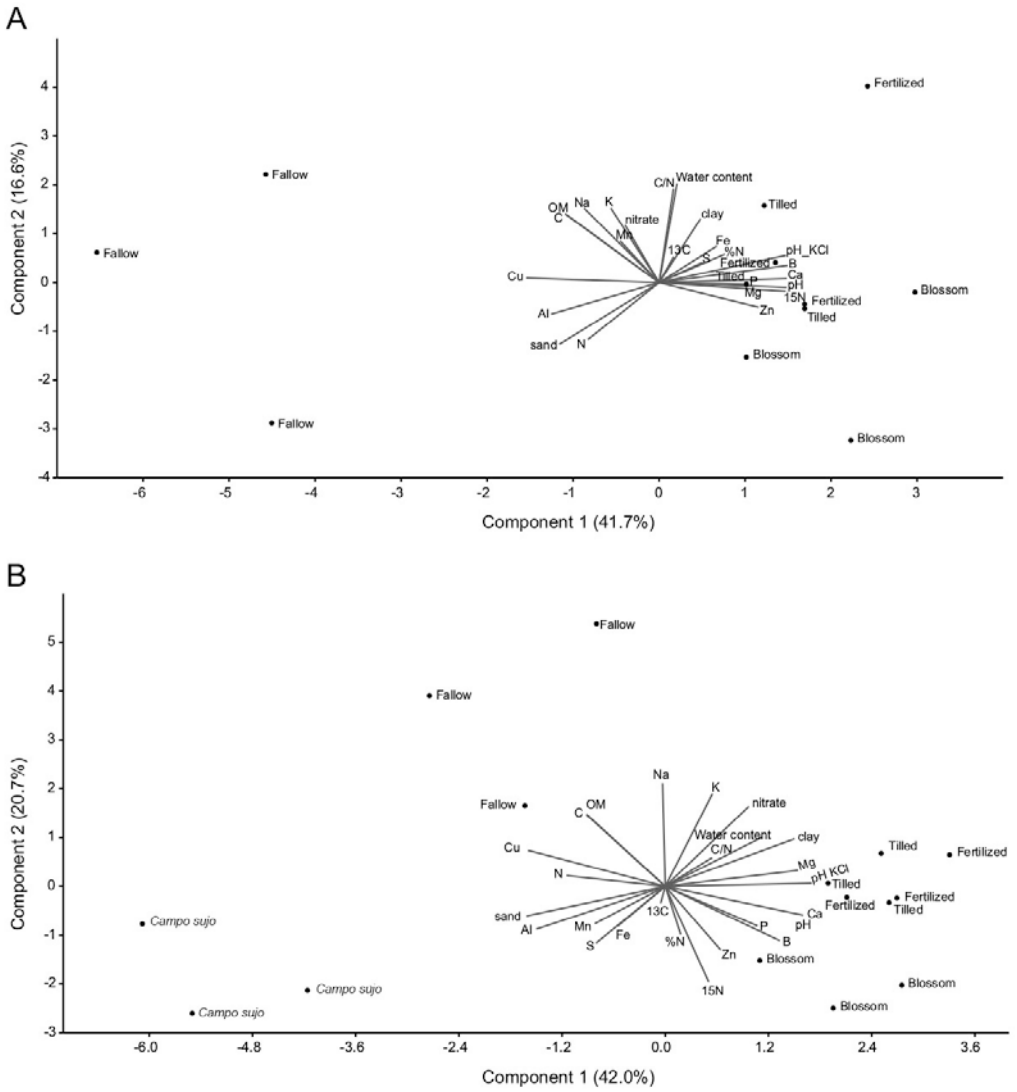


Figure 12. Principal component analysis (PCA) of soil physicochemical properties based on a correlation matrix performed in PAST v.3.01 (Hammer *et al.*, 2001). (A) Analysis of soybean site samples; (B) all samples including soil from the undisturbed Campo sujo site. Each vector points in the direction in which the respective value increases.

Ammonium and nitrate concentrations and soil $\delta^{15}N$

NH_4^+-N concentration in the undisturbed Campo sujo soil generally ranged from 5 to 8.3 µg g^{-1} dry soil, with two outliers of 11.8 and 48.7 µg g^{-1} dry soil (Figure 12A). The potential net N mineralization rate, determined by incubation of soil in the laboratory at room temperature, indicated that NH_4^+-N was becoming available in these soils at a rate of 0.8 to 3.29 NH_4^+-N µg g^{-1} dry soil day^{-1} (Figure 12C).

NH_4^+-N concentration was higher than NO_3^--N concentration in every soil sample but was particularly high in the undisturbed Campo sujo soil (Figure 12E). Fallow, tilled, and fertilized soils of the soybean site had similar average NO_3^--N concentrations, which were

higher than that of the bulk soil and rhizosphere soil collected during the blossom stage (Figure 12B). Nitrification was greater in fallow soil from the soybean site than in undisturbed Campo sujo soil (Figure 12D). Analysis of the soybean site samples showed a decrease in NH_4^+-N concentration as the crop developed, with significantly lower concentration in tilled soil and soil collected during the blossom stage of soybean development (both bulk and rhizosphere soils) than in fallow soil (Figure 12A). Nitrogen immobilization was greater than mineralization in fallow soil, recently tilled soil, bulk soil during the blossom stage, and especially in soil collected 1 month after fertilization. Nonetheless, the average net N mineralization differed significantly only between fertilized soil and soil collected during the blossom stage (both bulk and rhizosphere soils) (Figure 12C). Because fertilization was carried out at the same time as sowing, plant growth may have influenced the results obtained from soil collected 1 month after fertilization through NH_4^+-N uptake and the low inorganic N content in soil collected during the blossom stage. However, net N mineralization and nitrification occurred in a plant-free soil bag under laboratory conditions; therefore, NH_4^+ would have been assimilated by microorganisms or oxidized to NO_3^- by nitrifiers.

Another informative parameter was the NH_4^+-N:NO_3^--N ratio, with the lowest ratio observed in tilled soil, emphasizing the need for mineral N by the plants and soil microbial community during the blossom stage (Figure 12E). Figure 12E also shows the high ammonium/nitrate ratio in the undisturbed Campo sujo soil.

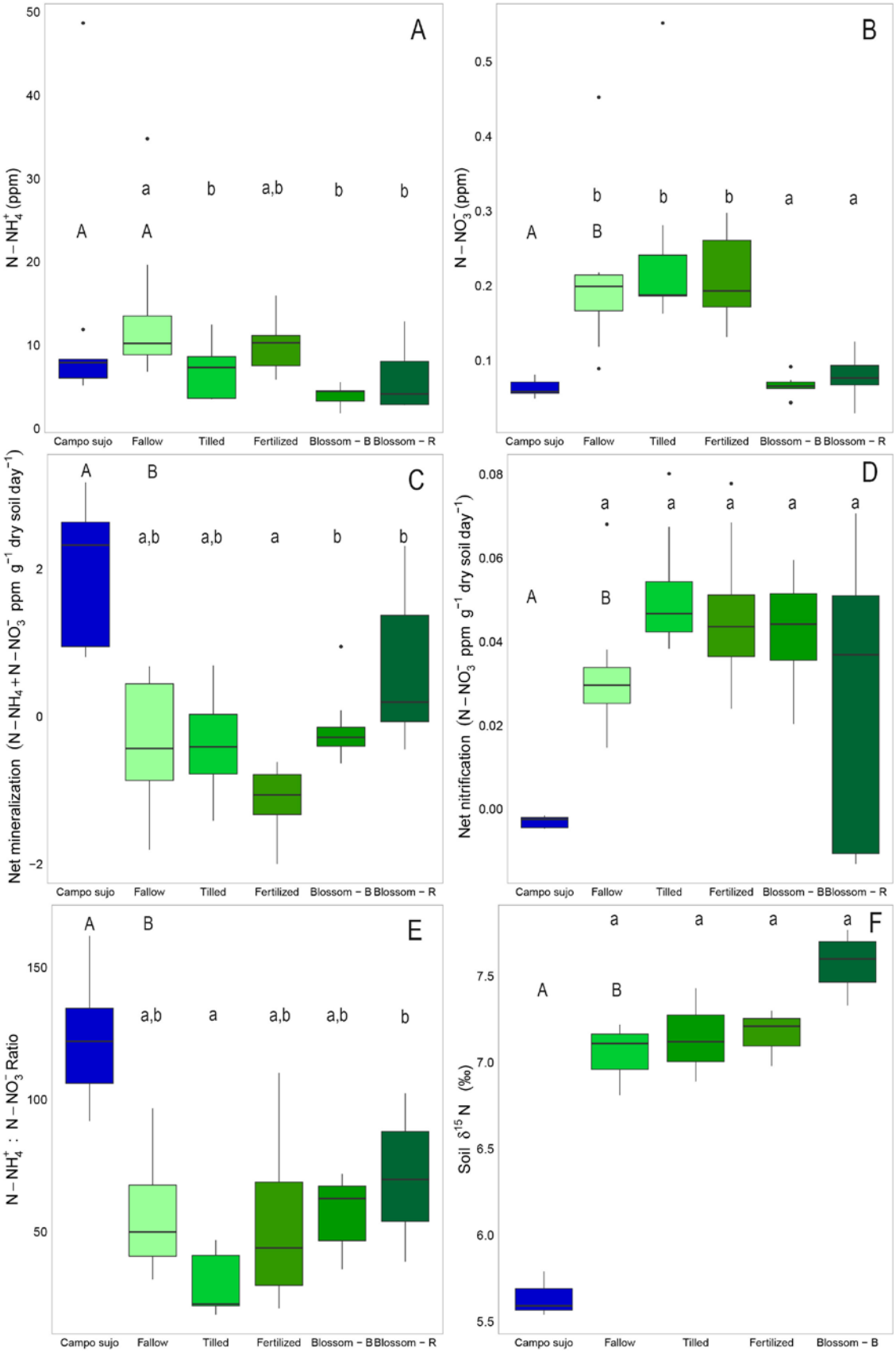


Figure 13. One-way ANOVA tests on soil N values, with Tukey–Kramer post hoc tests to compare group means (R with the ggplot2 package). Concentrations of (A) NH_4^+-N and (B) NO_3^--N in soil samples under each condition. (C) Net mineralization and (D) nitrification

determined by inorganic N and NO_3^--N content, respectively, measured after soil incubation in the laboratory for 1 week;(E) NH_4^+-N: NO_3^-N ratio and (F) integrated values of soil $\delta^{15}N$ (‰). Letters represent significant differences in inorganic N content between soil samples after post hoc tests: upper case letters represent difference between undisturbed Campo sujo and fallow soil from the soybean site; lower case letters present differences among soybean site samples. Soil samples obtained during the blossom stage of soybean development are represented by Blossom–B for bulk soil and Blossom–R for rhizosphere soil.

These results were supported by the integrated stable isotope ratios of C and N in these soils. The first soybean (C3 plant) cultivation did not change the $\delta^{13}C$ signal that remained from maize (C4 plant) cultivation or from the grassland before agriculture installation (Figure 13); however, the integrated soil $\delta^{15}N$ values were more labile. Soil $\delta^{15}N$ was significantly lower in the undisturbed Campo sujo soil than in fallow soil from the soybean site (Figure 12F). Although soil $\delta^{15}N$ did not significantly change during the soybean cultivation period, an increase was observed during the blossom stage (*p* value 0.0795, results of ANOVA between samples from the soybean site) (Figure 12F). These integrated isotope values are congruent with instantaneous values for mineralization and nitrification obtained from each sample in which significant changes in N cycle dynamics were observed, compared to the adjacent undisturbed site.

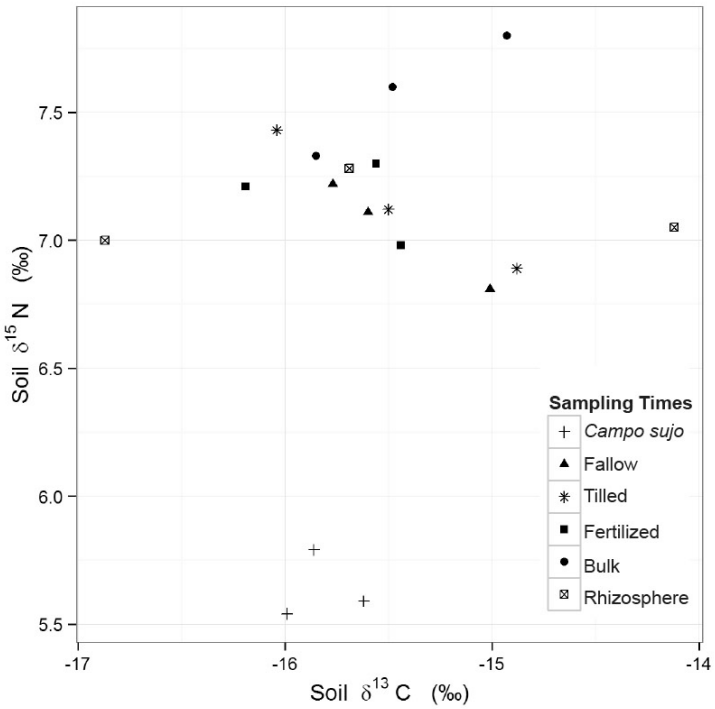


Figure 14. Relationship between soil $\delta^{13}C$ and $\delta^{15}N$ in ‰. Each point represents samples from each soil condition, marked with different symbols.

Abundance of archaeal and bacterial amoA genes

Archaeal 16S rRNA and archaeal and bacterial *amoA* genes were amplified with specific primers to quantify the abundance of these genes in the undisturbed site and in the soybean site.

The mean abundances of AOA and AOB *amoA* genes in the undisturbed Campo sujo site were 3.4×10^5 and 1.6×10^3 g^{-1} dry soil, respectively, representing an average AOA/AOB ratio of 212.9 (Figure 14C). In addition, AOA and AOB were, respectively, 26-fold and 49-fold less abundant in the Campo sujo site than the soybean site during the fallow period (Figure 14). The thaumarchaeal 16S rRNA:archaeal *amoA* gene ratio in the Campo sujo site varied from 785 to 1340 and was significantly higher than that of fallow soil from the soybean site.

The abundance of thaumarchaeal 16S rRNA and bacterial *amoA* increased during soybean development, but AOA *amoA* gene abundance decreased by 45% in the tilled soil compared to fallow soil. Tillage did not have the same effect on AOB, as demonstrated by the lack of significant change in AOB *amoA* gene abundance between fallow and tilled soil samples (Figure 14B). In fertilized soil AOA *amoA* gene abundance increased 2.6-fold and AOB *amoA* abundance increased 2-fold (Figure 14). However, AOB *amoA* gene abundance was more affected by soybean cultivation than AOA *amoA* gene abundance, as demonstrated by comparing rhizosphere soil with bulk soil during the blossom stage of soybean development. Furthermore, the increase in AOB abundance from fallow soil to rhizosphere soil was 2.9 greater than the increase in AOA abundance.

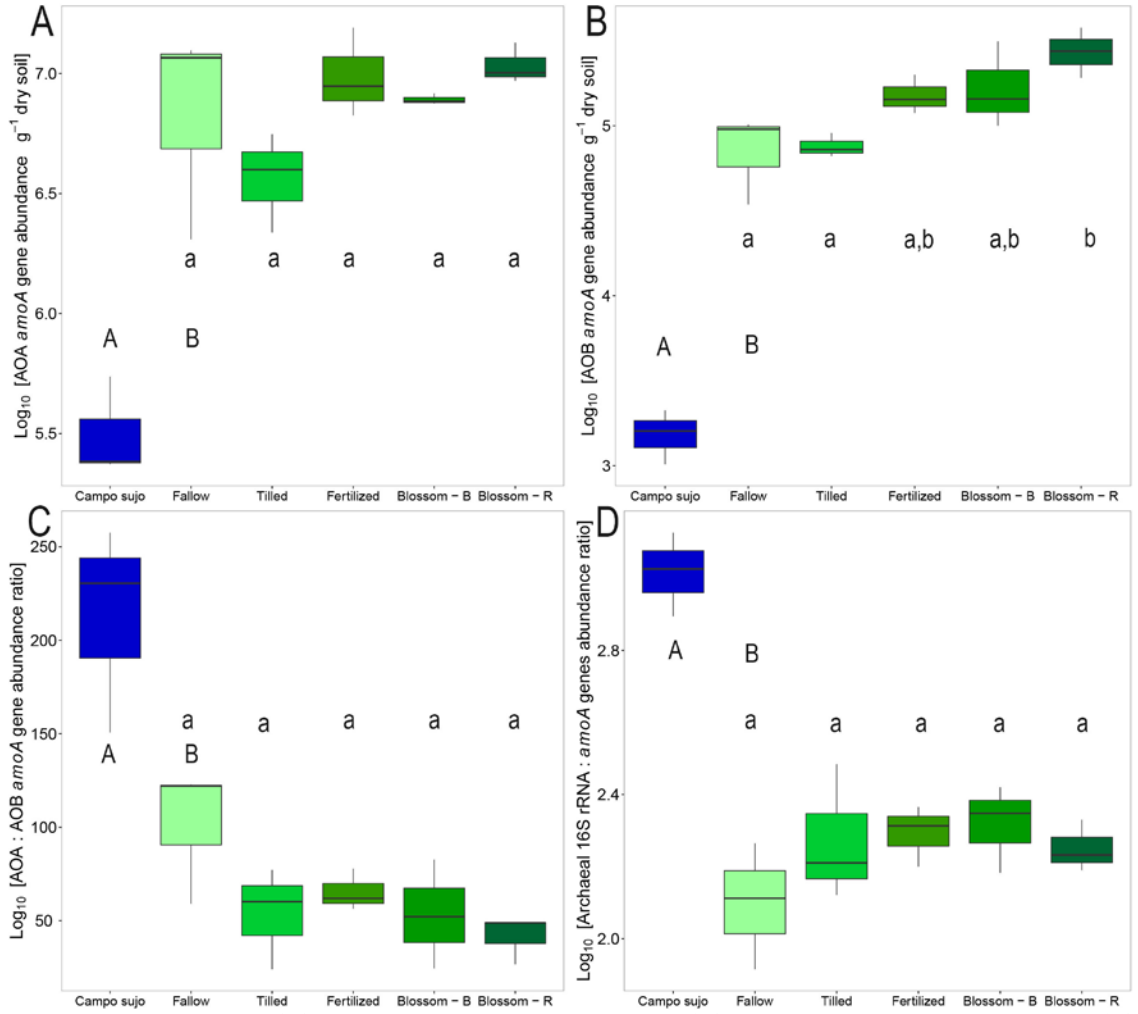


Figure 15. Changes in (A) AOA *amoA* gene abundance, (B) AOB *amoA* gene abundance, (C) AOA:AOB *amoA* gene abundance ratio, and (D) archaeal 16S rRNA:*amoA* gene abundance ratio. One-way ANOVA tests were performed, followed by Tukey–Kramer post hoc tests to compare group means (R package with the ggplot2 library). Different *letters* represent significant differences in gene abundance after post hoc tests: *upper case letters* represent difference between undisturbed Campo sujo and fallow soil from the soybean site; *lower case letters* present differences among soybean site samples. Soil samples obtained during the blossom stage of soybean development are represented by *Blossom–B* for bulk soil and *Blossom–R* for rhizosphere soil.

Soybean cultivation affected the abundance of both bacterial and archaeal ammonia oxidizers. The correlation between pH measured in H_2O and $\log_{10}$[AOB] (R^2 0.75, *p* value < 0.05 with the Bonferroni correction) was higher than the correlation between pH and $\log_{10}$[AOA] (R^2 0.63, *p* value < 0.05 with the BH correction). Similarly, the pattern of δ^{15}N was more strongly associated with $\log_{10}$[AOB] (R^2 0.96, *p*-value < 0.05 corrected by Bonferroni method) than with $\log_{10}$[AOA] (R^2 0.88, *p* value < 0.05 with the Bonferroni correction). Nevertheless, when analyzing only soils from the soybean site, AOA abundance did not correlate with pH, and the correlation between pH and AOB abundance was lower

(R^2 0.55, *p* value=0.72 with the Bonferroni correction). Similarly, the correlation between δ^{15} N and log_{10}[AOA] was not significant (R^2 0.24, *p* value=0.64 corrected by BH method) when analyzing only soils from the soybean site, but the correlation was still significant between δ^{15} N and Log_{10}[AOB] (R^2 0.68, *p*-value < 0.05 with the BH correction).

Discussion

In assessing links between environmental characteristics, nitrification, and the abundance of ammonia-oxidizer communities in the soil, it is important to assess abundances of both AOA and AOB, given the predominance of AOA *amoA* genes in many soils (Isobe 2012; Leininger 2006; Prosser and Nicol 2012). To assess the impact of land use conversion to soybean cultivation, ammonia oxidizer abundance and nitrification were evaluated in a soybean site after fallow, tillage, and fertilization and during the blossom stage of soybean development. These measurements were compared with those of an adjacent undisturbed Campo sujo site with low nitrate concentration, which is typical of Cerrado soil. These measurements support our hypothesis that both fertilization and soybean cultivation decrease the AOA/AOB ratio in association with increases in pH (Nicol et al. 2008; Prosser and Nicol 2012) and inorganic NH_4^+ (Levičnik-Höfferle et al. 2012), which is consistent with studies reporting that AOA are predominant in low-nutrient, low-pH environments (Erguder et al. 2009; Prosser and Nicol 2012). However, this study highlights the rapidity of changes in nitrifiers, N dynamics, and yields that occur in Cerrado soils after conversion to soybean cultivation.

The cultivation of soybeans in Brazil has been successfully implemented with inoculation of *Bradyrhizobium* strains to decrease or even completely eliminate the need for N fertilizers (Mendes et al. 2003). Nevertheless, the soybean site studied here required tillage and fertilization. Our results showed the effect of plant cover during the fallow period on soil recovery in the soybean site. Soil collected during the fallow period had soil characteristics similar to those of the undisturbed Campo sujo site, despite the different soil texture.

The undisturbed soil had the highest net N mineralization rate (average of 2 μg NH_4^+-N g^{-1} dry soil day^{-1}) and the lowest net nitrification rate, suggesting the inhibition of nitrification or low abundance of nitrifiers despite the presence of NH_4^+-N. However, potential nitrification was negative, indicating that the microbial community used nitrate at a faster rate than it was produced by nitrification. The soil was incubated in plastic bags; nitrate loss through leaching is negligible. Denitrification is unlikely at the moisture content of the soil used, and previous studies report that the loss of N gases is undetectable in undisturbed Cerrado soils (Bustamante et al. 2006; Pinto et al. 2002).

Both NH_4^+-N and NO_3^--N concentrations were particularly low in the soybean site during the blossom stage of soybean development, possibly because of N uptake by the

soybean plants. N mineralization exceeded immobilization in the rhizosphere soil but not in the bulk soil, which suggests greater N availability due to symbiotic N fixation. The soil C:N ratio > 20 (data not shown) in the bulk soil may partly explain the greater N immobilization, leading to depletion of N by both microbiota and plants. The decrease in NH_4^+-N and NO_3^--N during soybean growth was expected and is associated with periods of intense plant growth (Cruvinel et al. 2011). Nevertheless, Cruvinel et al. (2011) reported higher concentrations of NO_3^--N (1–52 mg kg^{-1}, depending on the period) and NH_4^+-N (21.3–50.7 mg NH_4^+-N kg^{-1} soil) in soils during soybean cultivation higher than the levels of NO_3^--N and NH_4^+-N concentration in the soybean site in recovery, supporting our finding that the soils sampled in our study were relatively depleted in mineral N. Cruvinel et al. (2011)also discussed possible competition between plant roots and microorganisms in the planted rows during cotton cultivation in the Cerrado because of the lower inorganic N availability and NO-N fluxes than that observed between rows. Low abundance of AOA and AOB in Cerrado soils may be due to competition with soil fungi for ammonium or inhibition by bioactive compounds synthesized by fungi (Yu et al. 2014). Nardoto and Bustamante (2003) showed that in both burned and unburned Cerrado areas, inorganic N content decreases during the rainy season, despite the observed increase in net N mineralization and net nitrification after the first rainfall events of the dry season (Nardoto and Bustamante 2003). These studies are consistent with our findings, as soils have higher levels of ammonia than nitrate, and the ammonium:nitrate ratio was lowest in the tilled soil, likely due to N release from organic matter. Similarly, the ammonium:nitrate ratio is high in integrated agricultural systems in Cerrado but is lower in crop-livestock and crop-livestock-forest systems compared to agroforestry and exotic pasture (Carvalho et al., personal communication). The same study also reports higher N_2O emissions from all of these agricultural systems compared with native Cerrado soils, with crop-livestock having the highest levels (Carvalho et al., personal communication).

Despite lower soil nitrate concentrations than those reported by other studies, N losses from the soybean site compared with the undisturbed Campo sujo site are suggested by higher $\delta^{15}N$ values and greater nitrate accumulation in the managed system. The integrative soil $\delta^{15}N$ signal, which provides historical information on soil N dynamics, indicates that soybean cultivation affects soil N accumulation, as the expected values for symbiotic N fixation were lower, at 0–2‰ (Delwiche et al. 1979). Nonetheless, the results demonstrate the labile characteristics of N compared to C, as $\delta^{15}N$ tended to increase during soybean cultivation, changing the short-term N dynamics in the cultivated soil,

whereas no significant changes in $\delta^{13}C$ were observed. A recent study reported that the $\delta^{15}N$ signature reflects a strong pattern of change according to land use, mainly due to soil C dynamics and clay content (Craine JME 2015).

Many soil characteristics are associated with changes in soil nitrification, including pH (Gubry-Rangin et al. 2011; Nicol et al. 2008), NH_3 and NH_4^+ concentration (Levičnik-Höfferle et al. 2012; Stopnisek 2010), O_2 (Erguder et al. 2009), temperature (Tourna 2008), soil moisture (Placella and Firestone 2013; Thion and Prosser 2014), and organic C (Erguder et al. 2009); however, pH and ammonia concentration have received greatest attention as potential drivers of ammonia oxidizer communities (Prosser and Nicol 2012). Kinetic studies of ammonia oxidation by *Nitrosopumilus maritimus* suggest that AOA have a higher affinity for ammonia (Martens-Habbena et al. 2009), but AOA may also be more sensitive than AOB to inhibition by high ammonia concentration (Prosser and Nicol 2012). In terms of pH, there is strong evidence for the selection of AOA, rather than AOB, in acid soils (Gubry-Rangin et al. 2011; Nicol et al. 2008; Zhang et al. 2012). However, AOA also contribute to nitrification in soils with pH > 5.5 (Gubry-Rangin et al. 2011; Gubry-Rangin et al. 2010), and there is evidence for long-term pH selection of both AOB and AOA phylotypes in soil (Nicol et al. 2008; Stephen et al. 1998). The increased pH observed during soybean cultivation was associated with a lower AOA:AOB ratio in our study, but no significant effect on nitrification was detected, and the expected decrease in pH that frequently accompanies nitrification was not observed. This may be due to liming or the low rates of ammonia oxidation observed in these soils. Therefore, pH may limit ammonia oxidizer growth in these low-nitrate Cerrado soils.

In this study we observed that tillage, fertilization, liming, and soybean monoculture altered soil pH, moisture, and inorganic N contents, all of which can influence the abundance and diversity of microbial communities and their functional potential, thereby influencing the production of nitrate, nitrite, NO, and N_2O (Mao et al. 2011). The change in land use had differential effects on the abundance of AOA and AOB communities, reinforcing the idea that these two microbial groups have distinct ecological niches associated with environmental variables. Specifically, samples from recently tilled soil and soil collected from the rhizosphere had smaller AOA:AOB ratios, and AOB showed a greater response to changes occurring during soybean cultivation. The lower abundance of AOA in undisturbed soil can be also related to the higher thaumarchaeal 16S rRNA:archaeal *amoA* ratio, which, in the absence of primer bias, indicates a great abundance of non-ammonia–oxidizing *Thaumarchaeota* (e.g., belonging to group 1.1c) (Weber et al. 2015).

A recent metagenomic study reported that *Thaumarchaeota* representatives were more abundant in no-till soils than in soils under conventional tillage (Souza *et al.*, 2013), possibly because of greater organic matter content or sensitivity to tillage. Although the AOA *amoA* gene was more abundant in all of our soil samples, the increase in AOB *amoA* abundance in tilled soil was greater. This finding may reflect the disruption of soil structure and release of C and N substrates previously not available to the microbiota.

Our results provided evidence for our hypothesis that both AOA and AOB abundance increase during soybean cultivation, with AOB increasing more than AOA, as predicted. Although AOA were more abundant, nitrification was better explained by the increase in AOB abundance, as predicted by the current view that AOB contribute more to ammonia oxidation than AOA in fertilized oxic soils at near-neutral pH. Wertz *et al.* (2012) reported an increase in AOB abundance with fertilizer application and nitrification in pine forests (Wertz et al. 2012).AOB abundance was more highly correlated with potential nitrification (Meyer et al. 2014), indicating that other factors can influence ammonia oxidizer communities. Moreover, although AOA abundance is potentially stable during the cultivation of bioenergy crops (*Zea mays* and *Miscanthus giganteus*), AOA diversity decreases, and AOB abundance increases, with this differential response to fertilization by AOA and AOB observed even 2 years after the fertilization (Mao et al. 2011).

A similar increase in the abundance of AOB, rather than AOA, was reported for a fertilized maize crop (Mao et al. 2011), and Mendes *et al.* (2014) recently showed that soybean plants select for the rhizosphere a specific subset of the soil bulk microbial community, which appears to be related to growth promotion and nutrition (Mao et al. 2011; Mendes 2014). Further studies are required to elucidate the differential effect of soybean cultivation on AOA and AOB abundance to determine whether these differences are direct effects of the soybean plant or due to fertilization promoting the growth of AOB.

Chapter 4 – Ammonia oxidizers in a non-nitrifying Brazilian savanna soil[4]

"Guid gear comes in sma' bulk."
Scottish saying

Abstract

Nitrification rate in tropical Brazilian savanna (Cerrado) soil is low to undetectable, puzzling researchers for decades. It was proposed that inhibitors in these soils, potentially produced by plants, could hamper ammonia oxidation. Recently we linked the absence of nitrification in an undisturbed Cerrado soil to low soil pH and a significantly lower abundance of archaeal (AOA) and bacterial (AOB) ammonia oxidizers than an adjacent Cerrado site changed to agriculture management. We also hypothesized that rain after the dry season allows higher microbial activity, including organic nitrogen mineralization and subsequent ammonia oxidation. To test these hypotheses, we (i) manipulated moisture and pH in microcosms containing Cerrado soil and (ii) tested nitrification inhibition in slurries assembled with a mixture of Cerrado and agricultural soil known for actively oxidizing ammonia. Very little NO_3^- accumulation was observed in Cerrado microcosms with either increasing moisture or pH, despite high ammonia concentration. In the Cerrado slurries, AOA *amoA* transcripts were detected after 14 and 21 days but not in all replicates. Besides, nitrification was not inhibited in the mixed soil slurries, final NO_3^- content being proportional to initial agricultural/Cerrado soil ratios, indicating a dilution of the ammonia oxidizer community, but no inhibition. In addition, DGGE profiles of the AOA community were similar in the mixed and nitrifying soils. Together, these results suggest that neither water availability, ammonia availability, low pH nor inhibition by soil compounds constrained nitrification in Cerrado soils. This distinctive pattern, i.e. the absence of nitrification despite the presence of AOA and AOB, might be associated with a particular community, specialized in high N immobilization in organic matter rather than in N loss through nitrification.

[4] Catão, E. C. P.; Thion, C.; Prosser, J. I. & Krüger, R. H. (2016) Ammonia oxidizers in a non-nitrifying Brazilian savanna soil. To be submitted to FEMS Microbiology Ecology.

Introduction

Nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a major cause of N loss in terrestrial environments, especially in agricultural systems, where 95% of total N is transformed through nitrification and denitrification, potentially leading to nitrate (NO_3^-) leaching and emission of nitric (NO) and nitrous (N_2O) oxides. In these systems the use of synthetic inhibitors of nitrification decreases nitrogen losses (Powell and Prosser, 1992). These inhibitors target the first step in nitrification, ammonia oxidation, which is carried out by both bacterial and archaeal ammonia oxidizers. In contrast, some natural systems have lower rates of nitrification and higher nitrogen use efficiency than managed systems (Ste-Marie and Paré, 1999). An example is the tropical savanna biome in Central Brazil, also called Cerrado, which has low to undetectable NO_3^- concentration (Nardoto and Bustamante, 2003), high NH_4^+:NO_3^- ratio and low abundance of nitrifiers (Catão *et al.*, 2016). These ecosystems may therefore provide a model for greater and more sustainable crop productivity and reduced demand for nitrogen fertilizers.

There are several potential explanations for low nitrification rates. Plants may reduce nitrification rates through competition for NH_4^+-N, supply of carbon from the plant, increasing C:N ratio and promoting higher rates of immobilization, or through inhibitory compounds in plant litter and root exudates (Subbarao *et al.*, 2006). More specifically, some plants release biological nitrification inhibitors (BNI) to the rhizosphere. These compounds target ammonia oxidation and reduce competition for ammonium by ammonia oxidizers (Subbarao *et al.*, 2006; Subbarao *et al.*, 2015), although the relatively high ammonium concentrations in Cerrado soil (3 – 22 ppm (Nardoto and Bustamante, 2003); 5 – 49 ppm (Catão *et al.*, 2016)) suggest that ammonia oxidizers are not limited by ammonia concentration. In addition, BNI-compounds are released by roots of plants grown with NH_4^+ but not with NO_3^- (Subbarao *et al.*, 2009), which could explain the inhibition of ammonia oxidation in the Cerrado soils.

Low rates of nitrification in acidic soils have been described for many years (De Boer and Kowalchuk, 2001) and rates often increase when acidic soils are amended with bases such as calcium carbonate (Fraps and Sterges, 1932), as also shown in Cerrado soil (Rosolem *et al.*, 2003). Inhibition of ammonia oxidation at low pH was traditionally considered to be due to the low availability of ammonia (NH_3), through ionization to NH_4^+, but may be alleviated in soil by growth in aggregates or on surfaces (Allison and Prosser, 1993; De Boer

et al., 1991), urease activity (Burton and Prosser, 2001; de Boer *et al.*, 1989) or through growth of acidophilic archaeal ammonia oxidizers (Gubry-Rangin *et al.*, 2011; Lehtovirta-Morley *et al.*, 2011) at low pH.

Low water availability also reduces nitrification rate (Placella and Firestone, 2013; Thion and Prosser, 2014). The Cerrado biome has well defined dry and wet seasons, and rainfall or artificial water addition results in N_2O pulses and 10-times more NO emission (Pinto *et al.*, 2006; Pinto *et al.*, 2002), leading to the hypothesis that ammonia oxidation can be limited during dry seasons in this biome.

Reasons for low nitrification rates in the Cerrado biome are unclear, but both archaeal and bacterial ammonia oxidizers are present in these soils (Catão *et al.*, 2016). The aim of this study was to test three hypotheses for potential mechanisms explaining low rates of nitrification. The first, the presence of plant-derived nitrification inhibitors, was tested by (i) analysis of the growth of cultures of ammonia oxidising bacteria (AOB) and archaea (AOA) in the presence of Cerrado soil aqueous extract, and (ii) by the effect of increasing amounts of Cerrado soil on ammonia oxidation by a nitrifying soil (Craibstone) in soil slurries. To second and third hypotheses, nitrification inhibition by low water availability or low pH, respectively), were tested by manipulation of Cerrado soil water content and pH in microcosms.

Materials and methods

Soil sampling

Triplicate soil samples were obtained from the upper 10 cm at each site and were pooled before sieving (2-mm mesh size) and stored at 4 °C. Cerrado soil was sampled from an undisturbed shrubland, termed Campo sujo, described previously (Catão *et al.*, 2016). The average monthly precipitation and temperature at this site, measured at the nearest meteorological center in 2014 (~30 km from the farm; Pirenopolis – GO, Station 83376, 15°50'60"S 48°57'36"W), were 143 mm (range 0 - 317 mm) and 23.4°C (range 21 - 25.6°C), respectively. The climate in the Cerrado biome is tropical (Köppen Aw) and samples were collected at the beginning of the dry season (May 2014). The soil, well-aerated and well-drained, is classified as sandy loam with 20.8% clay and had an initial pH of 5.6 (±0.04). Craibstone soil, used in this study as a reference nitrifying soil, was sampled from an experimental agricultural field (Scottish Agricultural College, Craibstone, Scotland, Grid reference NJ872104), maintained at pH 5.5 since 1961.

Cultures with or w/o soil aqueous extracts

Craibstone and Campo sujo soil aqueous extracts were prepared by blending 20 g soil in 2 volumes of sterile distilled water for 40 s and rotating in 50 mL sterile tubes for 1h. Aqueous extracts were then obtained by centrifugation (3,000 x g for 15 min) and sterilised by progressive filtration through 10-mm, 5-mm, 0.45-µm and 0.22-µm size pore filters. NH_4^+ and NO_3^- concentrations in the filtrates were below the level of detection (*data not shown*).

Pure strains of AOA (*"Candidatus Nitrosocosmicus franklandia"*) and AOB (*Nitrosospira briensis*, *Nitrosospira tenuis*, *Nitrosospira multiformis* and *Nitrosomonas europaea*) were cultivated in the dark without shaking, in inorganic growth medium. *'Candidatus Nitrosocosmicus franklandia'* (paper in revision) was cultivated at 40 °C in a previously described medium (Lehtovirta-Morley *et al.*, 2011) modified by addition of 1 mL L^{-1} vitamin solution (Widdel and Bak, 1992), 1 mL L^{-1} selenite-tungstate solution (Widdel and Bak, 1992) and 2 mM NH_4Cl. pH was maintained at ~7.5 by addition of 10 mL L^{-1} 1 M HEPES buffer. AOB were grown in SW medium (Skinner and Walker, 1961) at 30 °C. Triplicate cultures were prepared in 30 mL universal tubes by adding 5 mL of the appropriate medium previously inoculated with an exponentially growth culture (1 mL of innocula per 100 mL 2x concentrated medium) to other 5 mL medium of either sterile distilled water, Craibstone or Campo sujo soil aqueous extracts, or 100 µM allylthiourea (ATU) (final concentration), a

commercial ammonia oxidizer inhibitor. without agitation (Figure 15). Growth was monitored during 26 days (AOA) and 13 days (AOB) by measuring nitrite accumulation (Shinn, 1941) and maximum specific growth rate was estimated as the slope of semi-logarithmic plots of nitrite concentration vs time.

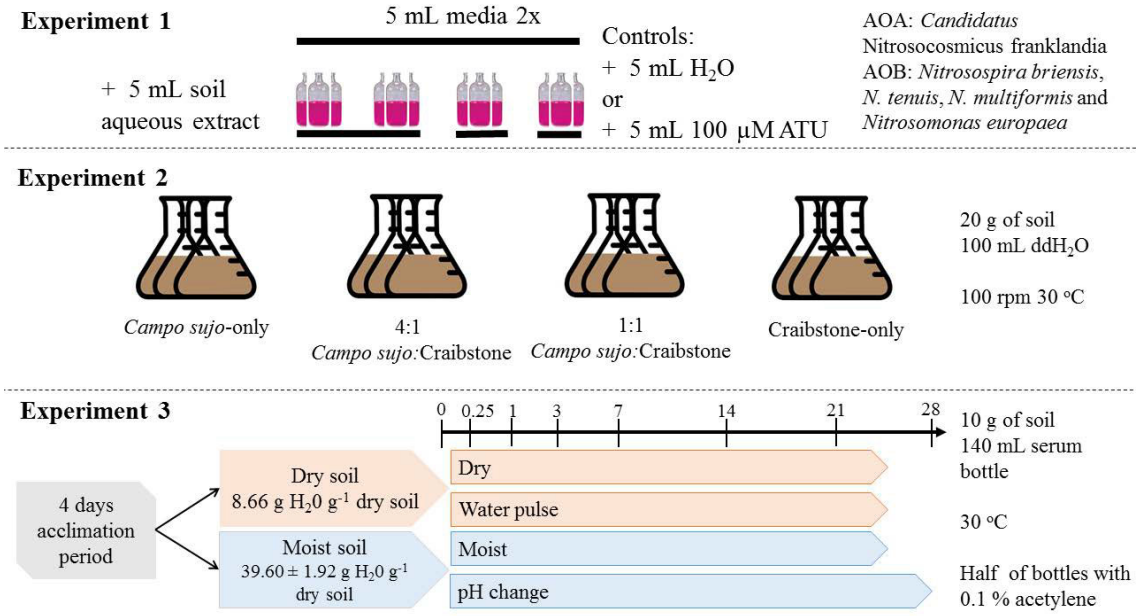


Figure 16. Graphical representation of the three experimental designs.

Soil incubation in slurries

Soil slurries were established in 250-ml sterile Erlenmeyer flasks containing 20 g soil and 100 mL sterile distilled water, stirred at 100 rpm and maintained at 30 °C in the dark (Figure 15). Flasks contained either Campo sujo soil, Craibstone soil or 1:1 or 4:1 ratios of Campo sujo and Craibstone soils. Soil slurry samples (8 mL) were centrifuged at 3,000 xg for 15 min. Supernatant (2 mL) was used for immediate measurement of pH, while the remaining supernatant (6 mL) was stored at -20 °C for quantification of inorganic N (see below). The soil pellet was frozen in liquid nitrogen and stored at -80 °C for nucleic acid analysis.

Soil incubation in microcosms

Cerrado Campo sujo soil was incubated in sealed microcosms consisting of 140-ml sterile serum glass bottles containing 10 g soil (Figure 15). Soil had an initial water content of 24.9 ± 0.03 g H_20 g^{-1} dry soil, corresponding to a matric potential of -0.15 ± 0.01 MPa. Microcosms were incubated for 4 days in the dark at 30°C (acclimation period), and then

divided in two groups. One group was left to air-dry, reaching a moisture content of 8.66 g H_20 g^{-1} dry soil (-6.34 ± 2.98 MPa matric potential), while the moisture content of the other was adjusted to 37.9 ± 0.3 g H_20 g^{-1} dry soil by addition of sterile distilled water. Soil in half of the 'dried soil' microcosms was rewetted to 39.6 ± 1.92 g H_20 g^{-1} dry soil (-0.11 ± 0.02 MPa) ('Water Pulse' treatment), while soil in the remaining microcosms was kept dry ('Dry' treatment). Finally, the pH of soil in half of the moist soil microcosms was increased to 6.34 ± 0.09 with $CaCO_3$ ('pH' treatment hereafter). The pH of soil in the remaining microcosms ('Dry', 'Water Pulse' and 'Moist' treatments) was 5.21 ± 0.02, which was slightly lower than the initial value of sampled soil, and was not adjusted. The four treatments were performed in triplicates, with or without addition of acetylene (0.01% of headspace volume). Microcosms were sampled destructively after 6 h and 1, 3, 7, 14 and 21 days (an additional time after 28 days was included for the pH treatment). For each microcosm, half of the soil was stored at -80°C for molecular analysis and the remaining soil was used for chemical analysis. Microcosms were incubated in the dark at 30°C and aerobic conditions were maintained by removing seals for 5 - 10 minutes twice weekly. 'Moist' and 'Water Pulse' microcosms were watered weekly to maintain moisture content.

Soil physicochemical analyses

Water matric potential was measured using a WP4C Dewpoint PotentiaMeter (Decagon, Pullman, UK) and pH was determined in water. Soil NH_4^+ and NO_x (NO_2^- + NO_3^-) concentrations were determined colorimetrically by flow injection analysis (FIA star 5010 Analyser, Foss Tecator AB, Höganäs, Sweden) (Allen, 1989) after extraction from 2 g of wet soil in 10 ml of 1 M KCl for the microcosm soil, or directly from slurry supernatant. As NO_2^- concentration was below the level of detection, NO_x is expressed as µg NO_3^--N g^{-1} dry soil (ppm). Inhibition was assessed as the percentage reduction in nitrate concentration in comparison to that of Craibstone soil at each time point.

Molecular analysis

Nucleic acids were extracted from 0.5 g soil as previously described (Nicol *et al.*, 2005), suspended in DEPC-treated water and immediately stored at -80 °C. cDNA was produced from an aliquot by DNAse treatment and RNA reverse-transcription as described previously (Tourna, 2008). Nucleic acid not used to for cDNA generation was considered as DNA only and the concentration was estimated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Loughborough, UK).

Archaeal and bacterial *amoA* genes were quantified in MasterCycler (Eppendorf), using standard curves as reference based on fragments obtained as described previously (Catão *et al.*, 2016) and primers crenamo23f and crenamo616r (Tourna, 2008) and bacterial *amoA* with amoA1F and amoA2R (Rotthauwe *et al.*, 1997), respectively. Each reaction had a final volume of 20 µl containing 1X QuantiFast (for AOA) or QuantiTect (for AOB) (Qiagen), 0.4 µM (AOA *amoA*) or 0.6 µM (AOB *amoA*) of each primer, 2 µg μl^{-1} BSA (Promega) and 2 µL of DNA (or cDNA). Archaeal *amoA* genes and transcripts were amplified according to the cycling conditions: 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 1 min 30 s at 60°C. AOB *amoA* genes were amplified using the following cycling conditions: 15 min at 95°C, 45 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C. SybrGreen fluorescence was measured after 5 s at 80 °C or 8 s at 83 °C, for AOA and AOB, respectively, to exclude fluorescence contamination of potential primer-dimers. Melting curves between 65 °C and 95 °C were analysed for each run. AOB *amoA* transcripts were below the detection limit (5 copies μl^{-1}). Efficiency of amplification and r^2 for DNA were, respectively, 0.92 and 0.998 for archaeal *amoA* and 104.6 and 0.993 for bacterial *amoA*.

AOA community composition in soil slurries was assessed by denaturing gradient gel electrophoresis (DGGE) analysis of *amoA* genes using the above primers in a linear gradient of 15 – 55% denaturant, as described previously (Nicol *et al.*, 2005).

Statistical analysis

All analyses were conducted using R version (3.2.2). The effect of soil aqueous extracts on pure AOA and AOB cultures was analysed by testing the difference between specific growth rates with a one-way analysis of variance (ANOVA) between treatments. The significance of differences between nitrification rates in soil slurries was tested using a linear mixed model (package *nlme*) (Pinheiro *et al.*, 2015) for repeated measures. Each slurry was considered a subject with random effect to analyse the effect of the fixed factors, i.e. treatment (mixed soil, Campo sujo or Craibstone slurries), time and their interaction, over the response variables: inorganic N concentration and *amoA* gene (and transcript) abundance. NO_3^- concentration in the Campo sujo slurries was below the limit of detection, and these samples were excluded from the analysis. Gene abundance data were log-transformed to achieve a normal distribution. When the interaction between the independent variables was not significant, it was removed to analyse the effect of time or treatment over the concentration independently. Two-way ANOVAs, with treatment and

time as independent factors, were performed to evaluate statistical differences in mineralization and NO_3^- in microcosms.

Results

Effects of soil extracts on ammonia oxidizer cultures

To assess the presence of potential nitrification inhibitors in soil, pure cultures of four AOB and one AOA were grown in liquid batch culture in medium amended with aqueous extracts. Soil aqueous extracts from both Campo sujo and Craibstone soils had no significant effect on the growth of any of the AO strains tested (Figure 16). Allylthiourea was used as a control for inhibition at 100 µM final concentration and completely inhibited all AOB cultures tested but interestingly did not inhibit growth of the AOA, *Candidatus* N. franklandia (Figure 16).

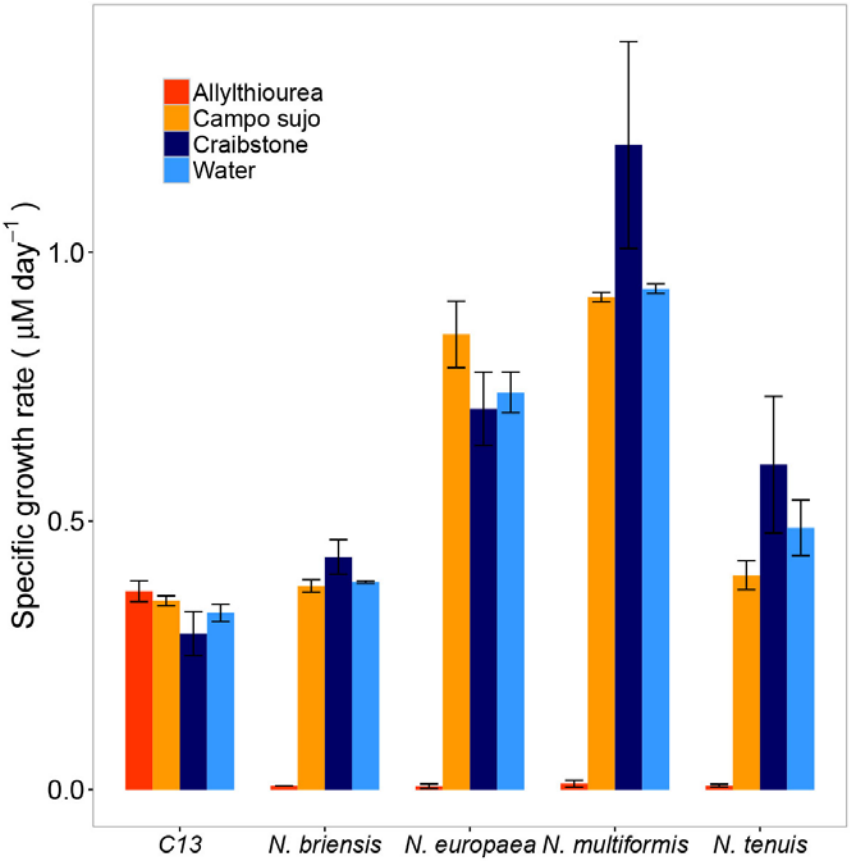


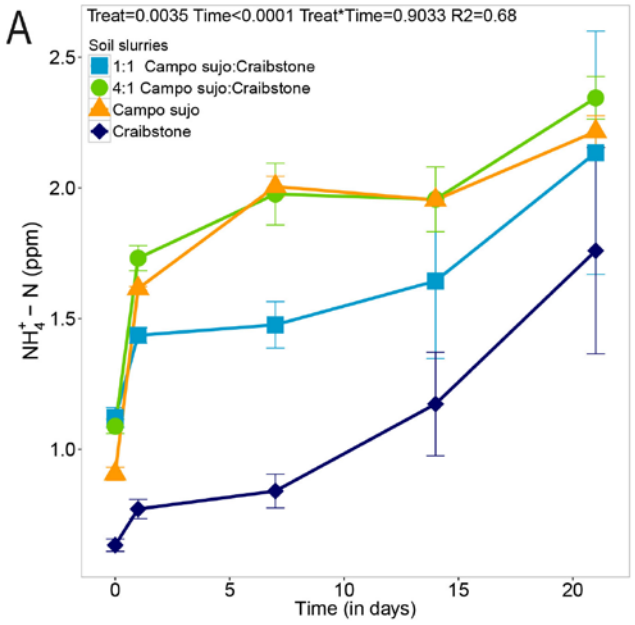
Figure 17. Specific growth rate calculated during exponential nitrite production batch cultures of the soil C13 and four soil AOB (*Nitrosospira briensis*, *Nitrosomonas europaea*, *Nitrosospira multiformis*, *Nitrosospira tenuis*) after addition of water (control), aqueous extracts of Campo sujo or Craibstone soil or 100 µM allylthiourea. Error bars represent standard errors of the means from triplicate cultures.

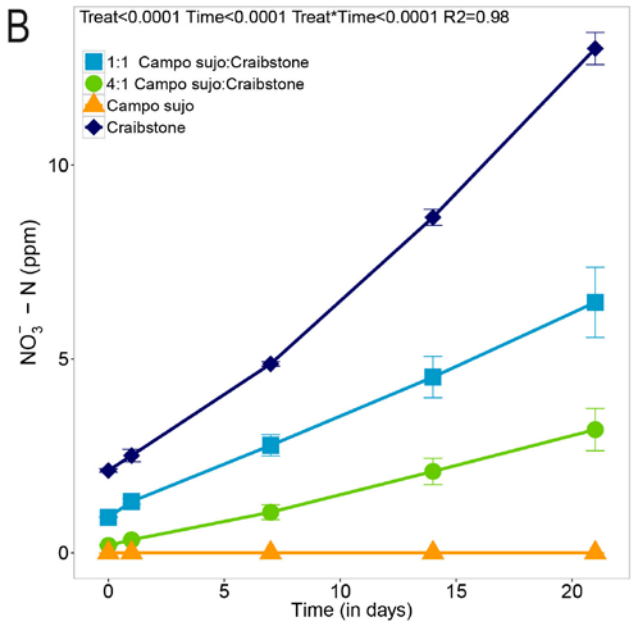
Effects of Campo sujo *soil on nitrification in Craibstone soil*

Soil slurries were established with mixtures of Campo sujo and Craibstone soils at ratios of 1:1 to 4:1, and with each soil alone, and were incubated in for 21 days. In all slurries, pH increased slightly after the first day of incubation, but did not change significantly during subsequent incubation.

Net NH_4^+ accumulation in the microcosms after 21 days ranged from 0.62 (±0.02) to 1.76 (±0.39) ppm for Craibstone and 0.87 (±0.02) ppm to 2.20 (±0.02) for Campo sujo (Figure 17). Ammonium concentrations were greater in mixed slurries than in controls, but accumulated less NH_4^+ during incubation, and the increase in NH_4^+ concentration after 21 days was greatest (2.9-fold) in Craibstone soil.

NO_3^- accumulated in all soil slurries (p<0.0001, Figure 17B) except those containing Campo sujo-only, in which no NO_3^- was below the detection limit. In the mixed slurries, NO_3^- production was equivalent or higher than the 50% and 20% expected for the 1:1 and 4:1 slurry (Figure 17C), thereby providing no evidence for inhibition of Craibstone soil nitrification by Campo sujo soil. Furthermore, the variance among replicate slurries (intra-treatment) was greater than 0, therefore significant, but smaller than the variance associated between subjects (inter-treatment) for both the ammonia and NO_3^- concentrations.


A
Treat=0.0035 Time<0.0001 Treat*Time=0.9033 R2=0.68
Soil slurries
1:1 Campo sujo:Craibstone
4:1 Campo sujo:Craibstone
Campo sujo
Craibstone
NH_4^+ – N (ppm)
Time (in days)


B
Treat<0.0001 Time<0.0001 Treat*Time<0.0001 R2=0.98
1:1 Campo sujo:Craibstone
4:1 Campo sujo:Craibstone
Campo sujo
Craibstone
NO_3^- – N (ppm)
Time (in days)

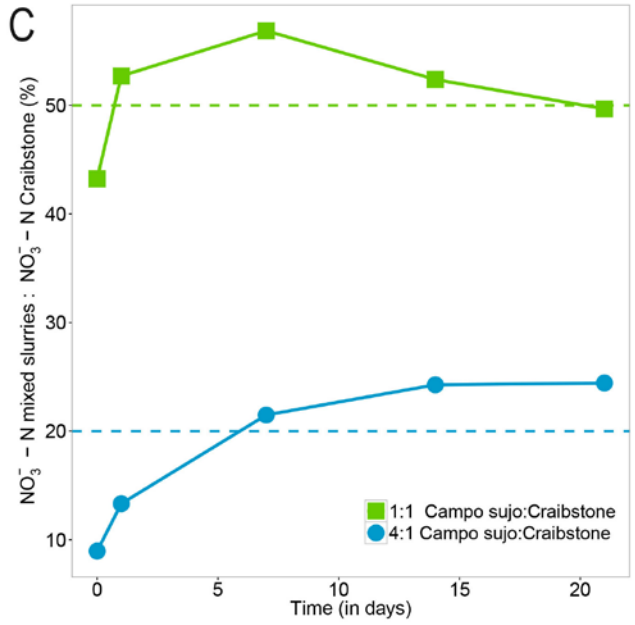

C
NO_3^- – N mixed slurries : NO_3^- – N Craibstone (%)
1:1 Campo sujo:Craibstone
4:1 Campo sujo:Craibstone
Time (in days)

Figure 18. Changes in inorganic N concentration during incubation of slurries of Craibstone and Campo sujo soils and mixtures of these soils. (A) NH_4^+-N concentration, (B) NO_3^-N and (C) NO_3^- concentration in mixed slurries as a percentage of that in Craibstone slurry. Statistical difference is shown by p value calculated with linear mixed model considering repeated measures (lme4 package, R version 3.2.3) for each independent variable and their interaction, and the marginal r^2 associated with the fixed effects. Error bars represent standard errors of the means from triplicate cultures.

There was no evidence for significant changes in ammonia oxidizer *amoA* abundance, even when significant NO_3^- accumulation was recorded (Figure 18). AOA *amoA* abundance in the Campo sujo-only slurries were approximately three orders of magnitude lower than in Craibstone-only slurries (Figure 18A). AOA *amoA* abundance in mixed slurries was lower than Craibstone-only until 14 days, when we could no longer detect significant difference between AOA *amoA* abundance between the mixed slurries and the Craibstone. Similarly, AOB abundance in the Campo sujo-only slurries was also approximately three orders of magnitude lower than in Craibstone-only slurries, except after 21 days, when abundance was not significantly different (Figure18B).

AOB *amoA* gene abundance was lower than AOA in all slurries and the AOA:AOB *amoA* gene ratio did not change in the Campo sujo-only slurries, in contrast to treatments with Craibstone soil, where the ratio increased (Figure 18C). AOB *amoA* transcripts were below the level of detection (5 copies μl^{-1}) in all slurries. AOA *amoA* transcripts were detected in all slurries containing Craibstone throughout incubation, but were only detected in the Campo sujo-only slurries after incubation for 21 days (Figure 18D).

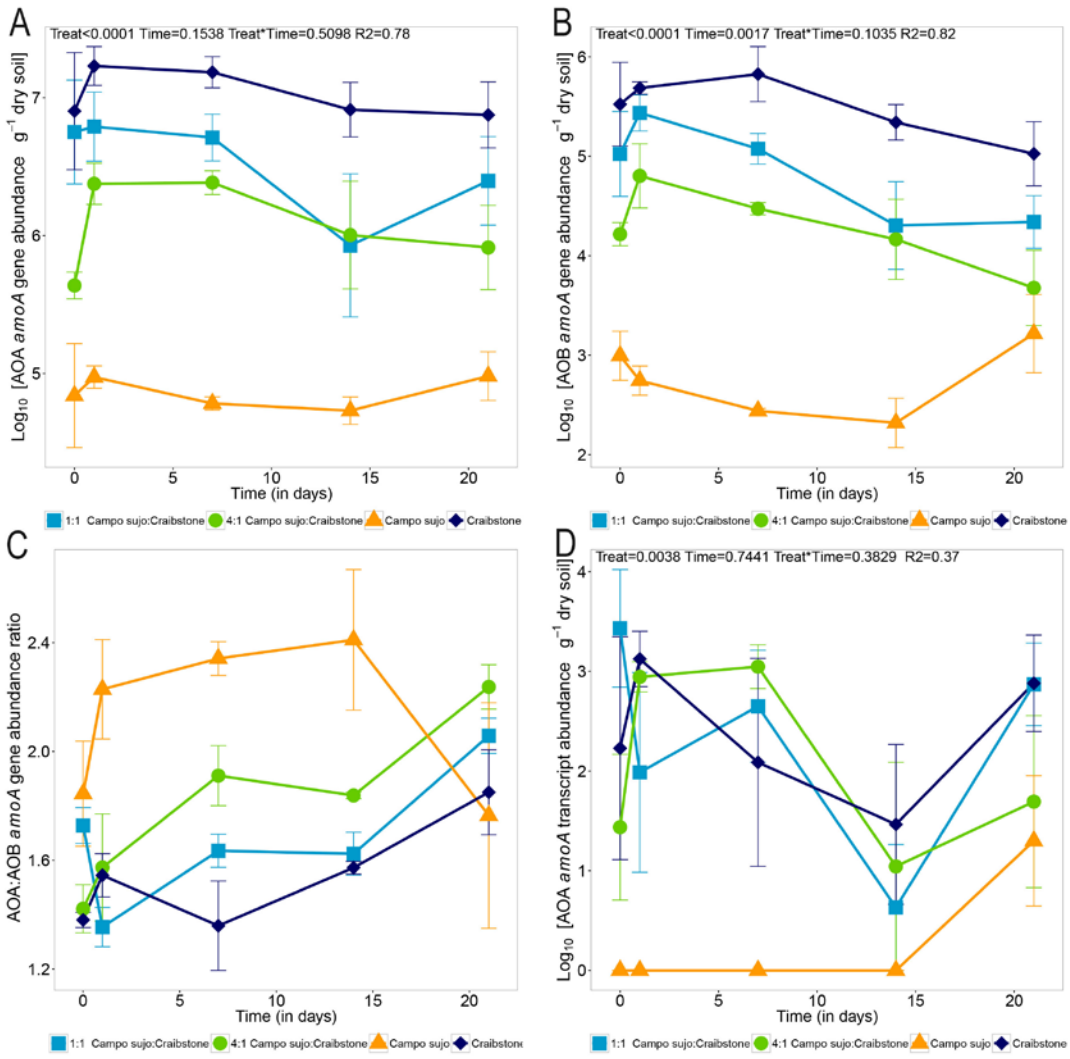


Figure 19. Changes in (A) AOA *amoA* gene abundance, (B) AOB *amoA* gene abundance, (C) AOA:AOB *amoA* gene abundance ratio and (D) AOA *amoA* transcript abundance during incubation of slurries of Craibstone and Campo sujo soils and mixtures of these soils. Statistical difference is shown by *p* value calculated with linear mixed model considering repeated measures (*lme4* package, R version 3.2.3) for each independent variable and their interaction, and the marginal r^2 associated with the fixed effects

AOA community composition was investigated by DGGE analysis of *amoA* genes and more DGGE bands were detected in DGGE profiles of Craibstone soil than Campo sujo soil (Figure 19), but will little evidence of changes in the AOA community during incubation. Total AOA community in the mixed slurry 1:1 was very similar to that of Craibstone soil as seen in the DGGE pattern of bands of AOA gene, possibly masking the presence of lower abundance of Campo sujo bands (Figure 19).

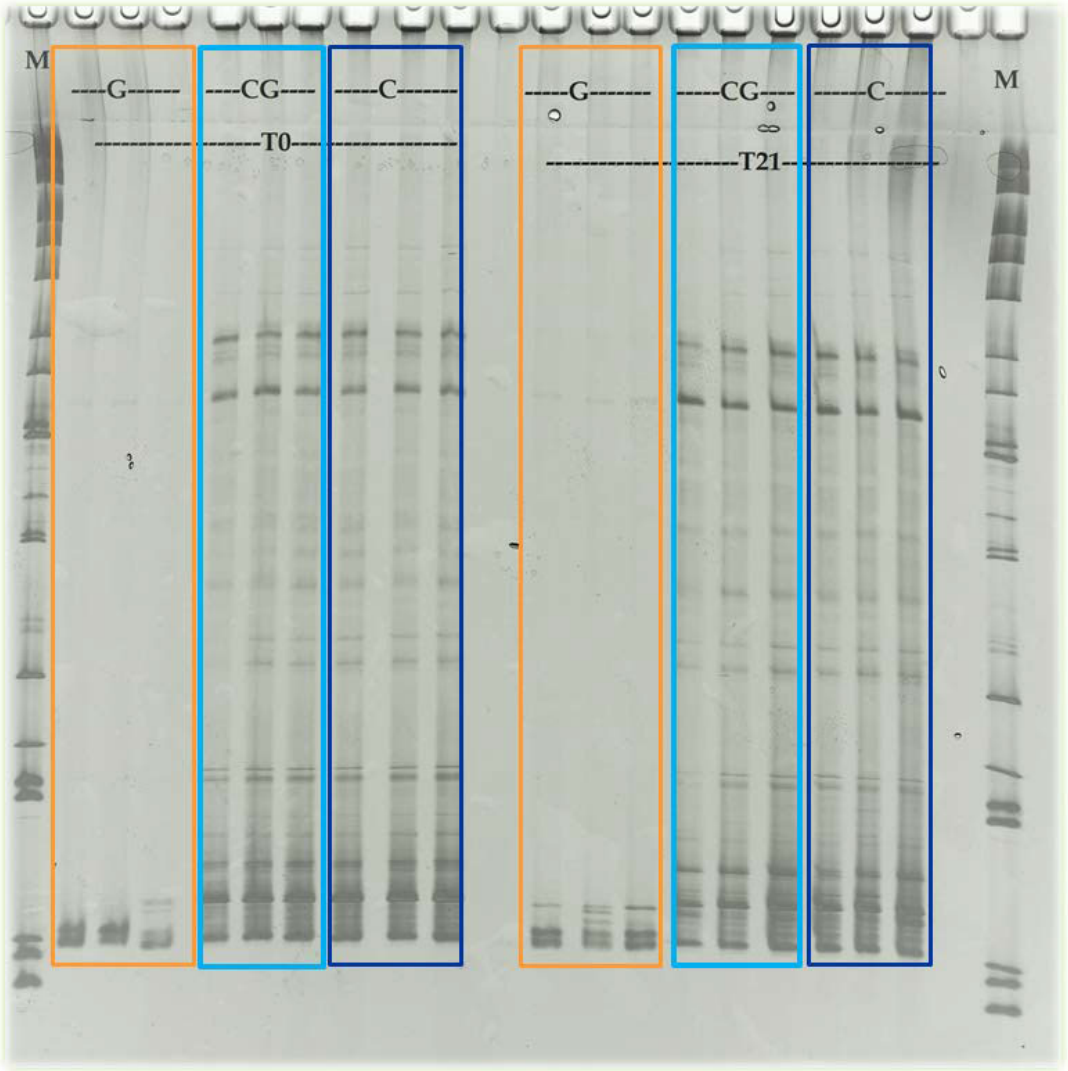


Figure 20. DGGE analysis of partial *amoA* gene products from triplicate soil slurries of (G) Campo sujo-only, (CG) 1:1 Campo sujo: Craibstone mixed and (C) Craibstone-only sampled after incubation for 0 and 21 days.

Effects of soil pH and moisture content

The effects of pH and moisture content on nitrification in the Campo sujo soil was investigated in soil microcosms. Mineralization in the microcosms was determined by the increase of inorganic N (NH_4^+-N + NO_3^- -N) concentration over time, assuming that other nitrogen cycle processes were not significant (Figure 20A). Mineralization in the dry soil did not increase after wetting, in contrast to the expected "Birch" effect (Birch, 1964)(Figure 20A). Soil pH did not change significantly with time in the microcosms and remained at 5.2 for 'Water Pulse', 'Moist' and 'Dry' treatments, and 6.3 for the 'pH' microcosms in which pH was increased artificially with $CaCO_3$. There was no evidence of significant increases in

nitrate concentration in any of the treatments (Figure 20B), with no significant difference between treatments (*p* treat=0.140).

Acetylene was used as an inhibitor of ammonia oxidation in half of the samples for all four treatments ('Dry', 'Water Pulse', 'Moist' and 'pH') to discriminate N utilization by soil ammonia oxidizers. No significant difference was observed with and without acetylene, except for the NO_3^- -N concentration in the moist microcosms after 21 days. After 21 days, NO_3^- -N concentration was higher in the non-acetylene treated moist microcosms than in those with added acetylene.

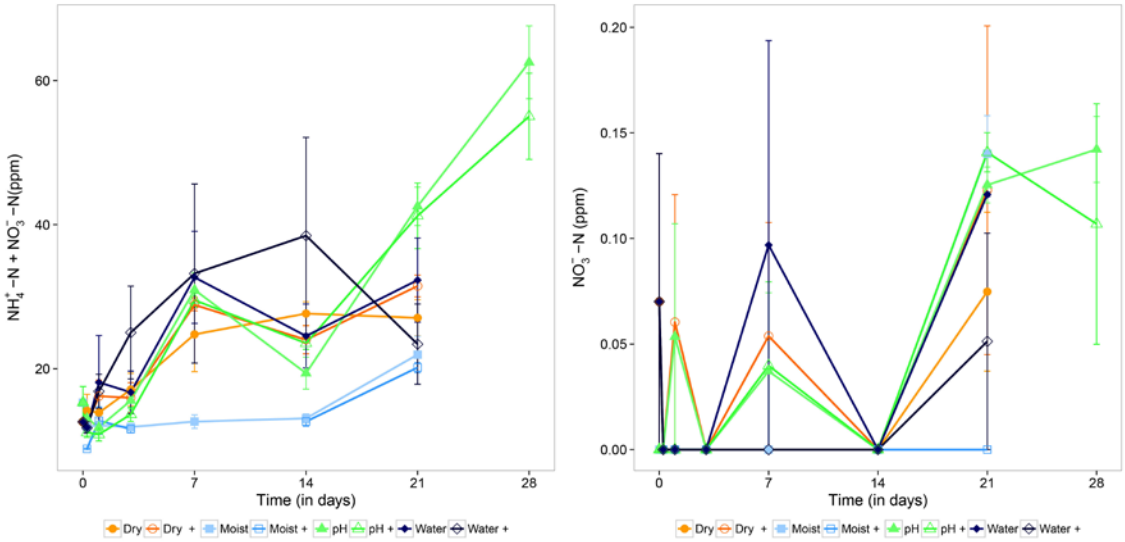


Figure 21. Changes in (A) (NH_4^+-N + NO_3^--N) and (B) NO_3^--N during incubation of microcosms containing Campo sujo or Craibstone soil after manipulation of pH and moisture content. Open symbols represent treatments with addition of 0.01% acetylene in the headspace. Red line represents the threshold limit of detection considered for the FIA technique. Dry: air-dried soil to 8.66 g H_20 g^{-1} dry soil; Water: rewetted soil to 39.6 ± 1.92 g H_20 g^{-1} dry soil; Moist: moist soil; pH: soil treated with CaCO3 with one pH unit higher than the other treatments.

Discussion

Nitrification is frequently undetectable in undisturbed Cerrado ecosystems, although its management and conversion to agricultural production results in nitrate production (Catão *et al.*, 2016). Previous studies provide evidence for low abundance of AOA and AOB in Campo sujo soil (Catão *et al.*, 2016), which is a shrubland with some sparse shrubs over a continuous grass layer. The aim of this work was to determine whether the lack of nitrification and low abundance of AO was due to low pH, low soil moisture or NH_4^+ limitation or biological inhibition of ammonia oxidation.

Certain plants release biological nitrification inhibitors (BNI), that suppress ammonia oxidation in soils (Subbarao *et al.*, 2015), and some, for example produced by *Brachiaria* (Subbarao *et al.*, 2009) and *Sorghum* (Zakir *et al.*, 2008), inhibit a recombinant *N. europaea* strain possibly by blocking the ammonia monooxygenase and hydroxylamine oxidoreductase enzymes (Subbarao *et al.*, 2008). Exudation of BNI can be promoted by exposure to high of NH_4^+:NO_3^- ratios (Subbarao *et al.*, 2015), which are found in Campo sujo soil (Catão *et al.*, 2016). There was, however, no evidence for nitrification inhibitors in the Campo sujo soil. Cultures of four AOB and one AOA, all of which were originally isolated from soil, were not inhibited by aqueous soil, where allylthiourea, a known inhibitor of AOB, prevented nitrification of these organisms, but not that of the AOA culture. This is consistent with other studies that indicate greater tolerance of AOA to allylthiourea (Hatzenpichler and Lebedeva, 2008; Stempfhuber *et al.*, 2015), highlighting the need to testing of potential BNI and other inhibitors against both AOA and AOB, rather than *N. europaea* only.

The above studies suggest the absence of inhibitors within Campo sujo soil, but are based on aqueous soil extracts and a small number of cultivated strains. The potential for soil inhibitory factors was tested more directly by mixing Campo sujo soil with Craibstone soil, a strongly nitrifying soil (Nicol *et al.*, 2008; Zhang *et al.*, 2010) with similar pH, in soil slurries. Soil slurries also provided no evidence of nitrification inhibitors in Campo sujo soil. Nitrate accumulation in soil mixtures was lower than in 'undiluted' Craibstone soil but reductions in mixtures were greater than or equal to those predicted merely through dilution, and not through additional inhibition. There was also no evidence for negative effects of Campo sujo soil on AOA and AOB *amoA* gene abundances. AOA *amoA* genes were more abundant than those of bacteria, and no bacterial *amoA* transcript was detected, as found in previous studies with Craibstone soil (Zhang *et al.*, 2010). Neither AOA nor AOB

amoA abundance changed significantly during incubation in any of the slurries containing Craibstone soil, despite active nitrate production, but there was evidence for an increase in the AOA:AOB *amoA* gene ratio, suggesting greater growth, or lower death, of AOA. There was no evidence for growth of AOB or AOA in the Campo sujo soil. The low abundance of *amoA* transcripts in the Campo sujo soil, and smaller number of DGGE bands, compared to Craibstone soil, are further evidence of the low abundance and activity of ammonia oxidizers in the former soil and the absence of detectable ammonia oxidizer activity. Nevertheless, AOA *amoA* transcripts detection in Campo sujo-only slurries after 21 days suggest that AOA had begun to grow in the Campo sujo soil but that their abundance was not sufficient for them to generate detectable nitrate. Alternatively, undetectable nitrate can reflect the greater variability of *amoA* community present in the Craibstone-only slurries and the mixed slurries.

Microcosm studies were performed to determine whether low nitrification rates were due to low pH or low soil moisture content. Soil pH is considered one of the major factors that influences microbial diversity (Fierer *et al.*, 2012a; Lauber *et al.*, 2009); it has previously been shown to influence soil ammonia oxidizer abundance and activity (de Boer and Kowalchuk, Nicol et al. 2008), with higher transcriptional activity of *Archaea* than Bacteria as pH decreases (Nicol *et al.*, 2008). An increase in soil pH increased the mineralization rate but did not lead to detectable nitrate production in Campo sujo soil after incubation for 28 days. There was therefore no evidence for limitation of nitrification by low pH. Mineralization was lower in moist soil, but again, the increase in moisture did not lead to detectable nitrate production.

Low nitrification, and low AO abundance, in both microcosms and slurries was not due to NH_4^+ limitation, as NH_4^+ concentration was even higher than that measured in the Craibstone soil slurries at the beginning of the experiment. Ste-Marie and Paré (Ste-Marie and Paré, 1999) described similar results on a jack pine forest soils that accumulated high concentrations of ammonium but nitrate was not detectable. None of the treatments applied promoted nitrification in Campo sujo soil and no inhibition by this soil on Craibstone or pure culture nitrifiers was observed. In the jack pine forest, nitrification was not stimulated by an increase in pH or ammonium amendment, but by the addition of nitrifying soil from a forest floor (Ste-Marie and Paré, 1999). In our study, both AOA and AOB were detectable, but at low levels that are unlikely to lead to detectable nitrate production. As a consequent, Cerrado soils have much greater ability to retain N as NH_4^+, through ion exchange, and leaching NO_3^- of nitrate will be low. Our study indicates that low nitrification

rates and ammonia oxidizer abundance are not due to low moisture content, low pH or ammonia oxidizer inhibitors within the soil.

Some other process of NO_3^- use by the microbiota should be considered in further studies, i.e. competition for NH_4^+ by plants or heterotrophic microbes, as described in a Californian grassland (Jackson *et al.*, 1989); dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) (Chen *et al.*, 2015; Cole and Brown, 1980).

Chapter 5 – Abiotic and biotic factors that affect ammonia oxidizers and therefore nitrification: final discussion

"Le savant n'étudie pas la nature parce que cela est utile; il l'étudie parce qu'il y prend plaisir et il y prend plaisir parce qu'elle est belle"

Henri Poincaré

20 years, at least, have passed since N cycling is the focus of research in the Cerrado biome. Each experiment takes us one step ahead to understand N conservation in soil ecosystem and the changes that take place in N utilization by soil fauna, flora and microbiota after natural or artificial disturbances (i.e. fire episodes and agriculture, respectively). The relevance of understanding N dynamics in the biosphere is both related with how N limitation controls net primary productivity and the fact that N cycle is one of the most affected cycles by anthropogenic impact, having crossed the threshold for planetary boundaries (Rockström *et al.*, 2009). Yet, it is a cycle mainly controlled by microbial dynamics, therefore the numerous scientific researches focusing on the association of specific groups of microorganisms and the N metabolism.

In the Cerrado, microbial community structure varies according to the types of vegetation (Araujo *et al.*, 2012; Catão *et al.*, 2013; de Castro *et al.*, 2008; Quirino *et al.*, 2009), and to the variations in soil moisture that occurs in Cerrado soils (Catão *et al.*, 2014; Pereira de Castro *et al.*, 2016; Viana *et al.*, 2011). Recently, Pereira de Castro et al. (2016) discussed the general metabolic potential distribution in the Cerrado biome besides the taxonomy approach. Nonetheless, until now no work has focused on the microbial genes associated with N cycling in the Cerrado.

NO_3^-, the most oxidized form of N, does not accumulate in native Cerrado soils as described in the literature and as found in the three projects developed here (0.03-0.09 µg NO_3^--N g^{-1} dry soil). Furthermore, NO_3^- were low or undetectable in soils sampled from the Campo sujo, contrary to the values obtained in the soil under cultivation of soybean. Net nitrification, obtained as the NO_3^--N over time, was also insignificant in most of the manipulated soils microcosms and slurries, except in the pH and moist microcosms in which NO_3^- was detectable after 21 days of incubation. In all of the experiments here performed, NH_4^+:NO_3^- was always greater than 10, sometimes greater than 50 (as for example in the Cerrado *sensu stricto* of PNCV, and the Campo sujo in the Farm).

Low net nitrification in the Cerrado biome seems to be related with different biotic and abiotic factors, here described. Soil nutrient dynamics is neither unidirectional nor linear, even though most times the variables have to be considered as such. For instance, NH_3 is the substrate for nitrification, but it can be inhibitory in determinate concentrations, especially to AOA that seem to be more sensitive than AOB to ammonia inhibition (Prosser and Nicol, 2012). Therefore, to say that oxidation of ammonia by *Bacteria* or *Archaea* is dependent of the soil pH, or the quantity or quality of N substrate, or moisture, is not informative *per se*. Especially if we want to understand how soil characteristics' affect the microbial community (or vice-versa), and how we can have better manage the land use with less environmental pollution. This discussion focuses on the results obtained along this thesis and elaborates on the microbial ecology of nitrification, which is resumed in Figure 21.

pH

pH is within the most cited variables that explains bacterial community distribution in terrestrial ecosystems whether in local scale (Rousk *et al.*, 2010), regional (Bru *et al.*, 2011; Griffiths *et al.*, 2011; Kuramae *et al.*, 2012) or global (Lauber *et al.*, 2009). pH affects microbial cells direct or indirectly and different communities have optimal growth pH, as low pH seems to be more limiting for bacterial growth than for fungal growth (Bárcenas-Moreno *et*

al., 2016). As an example, acidophilic organisms require high protons concentration to keep the integrity of the cytoplasmic membrane, and bacterial communities from acidic soil reach their respiration peak twice later than communities from soil with higher pH (Bárcenas-Moreno *et al.*, 2016). Nonetheless, bacteria adapted to low pH grow more in higher pH, but bacterial communities transplanted from alkaline soil to acidic one are less successful to grow (Bárcenas-Moreno *et al.*, 2016). As a result, it is not surprising that we find a great correlation between soil pH and microbial distribution.

Archaeal communities, and more specifically archaeal oxidizers, also present ecological coherence with pH in soil and can be classified depending on the soil pH of their greatest occurrence (Gubry-Rangin *et al.*, 2011). AOA predominance over AOB (Leininger *et al.*, 2006) is often associated with low pH soils (Erguder *et al.*, 2009; Gubry-Rangin *et al.*, 2011; Lu *et al.*, 2012; Nicol *et al.*, 2008; Prosser and Nicol, 2012). This was also observed in our data where both fertilization and soybean cultivation decreased the AOA:AOB ratio in association with increases in pH. However, some AOA clusters contribute to nitrification in soils with pH > 5.5 (Gubry-Rangin *et al.*, 2011; Gubry-Rangin *et al.*, 2010), and there is evidence for long-term pH selection of both AOB and AOA phylotypes in soil (Nicol *et al.*, 2008; Stephen *et al.*, 1998). As different groups, either between AOA and AOB or within this clades, have different sensibilities to pH (Stempfhuber *et al.*, 2015) nitrification measurement should be estimated with original soil pH and not with changed pH in buffered potential nitrification assays as performed sometimes.

We have observed *in situ* the change in NO_3^- –N accumulation along the soybean cultivation possibly due to liming and associated with a decrease in the AOA:AOB gene abundance ratio. The field study performed in the farm at Cocalzinho de Goiás (GO) showed a rapid turnover (nearly 4 months) effect of the agricultural practices on the soil microbial community. Whether higher nitrification activity in the soybean soil is due to greater cell growth rates or higher NH_3 availability, both related with the greater pH, or if it is a consequence of the NPK solution and urea provided remains to be tested.

Increase in the abundance of ammonia oxidizers associated with increased pH in the soybean cultivation suggested a pH limitation for ammonia oxidizers growth in the Cerrado soils, which lead to the experiment of pH change in soil microcosms. The soil from the Campo sujo was mixed with $CaCO_3$ to increase pH in one unit in microcosms. During incubation it mineralized more inorganic N than the microcosms incubated with the original pH. Net nitrification increased significantly after 28 days of incubation, but nitrate accumulation was still little (0.14 ± 0.03 µg NO_3^--N g^{-1} dry soil). We cannot rule out the

impact of pH changing the soil microbial community associated with nitrate production in the Campo sujo soil, but modification possibly takes more than 1 month to be significant in laboratory or field assays.

On the other hand, the incubation of the Campo sujo soil in slurries with Craibstone soil of same pH (original pH of 5.3) in a ratio 1:1 accumulated nitrate due to the activity of ammonia oxidizers from the Craibstone soil. In 1932, Fraps and Sterges presented similar conclusion that soils with little ability to nitrify has increased nitrate concentration after either soil pH modification with calcium carbonate and/or with the addition of nitrifying soil (Fraps and Sterges, 1932).

Experimental liming in Cerrado parcels lead to a greater differentiation in the microbial community than in the treatments fertilized with N, P or N+P, with a special increase in certain phyla as Gemmatimonadetes (da Silva, 2012). In addition, an increase in NO_3^- -N concentration was observed most likely due to the increase in pH to 6 (initial pH was approximatively 4) (da Silva, 2012), which is expected since liming can increase organic matter mineralization a nitrification in soils (Rosolem *et al.*, 2003). Nevertheless, liming was performed in those areas for years which differs from our study in the microcosms that lasted only 1 month.

NH_4^+

Furthermore, cells need to be adapted to the availability of nutrients in lower pH. NH_3 instead of NH_4^+ is assumed to be the substrate for ammonia oxidation (Suzuki *et al.*, 1974) as NH_3 diffuses passively but NH_4^+ needs active transport inside the cell, which leaded to the hypothesis that intracellular urea hydrolysis facilitates autotrophic ammonia oxidation in low pH soil and NH_4^+ produced in excess can locally increase pH (Burton and Prosser, 2001).

High NH_4^+ concentration was considered to be toxic to AOA especially, which seemed to be more sensitive to NH_4^+ concentration than AOB (Verhamme *et al.*, 2011), and AOA were found in higher abundance in the Campo sujo (Catão *et al.*, 2016) soil as expected due to the low pH (Prosser and Nicol, 2012). As the undisturbed Campo sujo soil had the highest net N mineralization rate (average of 2 µg NH_4^+-N g^{-1} dry soil day^{-1}) and the lowest net nitrification rate, an inhibition of nitrification or low abundance of nitrifiers despite the presence of NH_4^+-N had to be considered. However, inhibition by NH_4^+ concentration was also ruled out as ammonia oxidizers community from Craibstone was also represented by more AOA than AOB gene abundance and activity and was stimulated by the NH_4^+

concentration with increased nitrification with time. Others have found similar result were NH4+ availability did not constrain net nitrification (Nugroho *et al.*, 2007).

Heterotrophic nitrification

Substrate is one of the determinants for the predomination in AOA or AOB in terms of ammonia oxidation: AOA dominate soils in which ammonia is available from organic N (Levičnik-Höfferle *et al.*, 2012) and in lower concentrations (Prosser and Nicol, 2012). The balance between autotrophic and heterotrophic nitrification seems also to be regulated by the substrate source in certain soils (Zhang *et al.*, 2014). Poth et al (1995) detected the NO formation related to hetetrotrophic nitrifiers in Cerrado (Poth *et al.*, 1995).

In addition, C substrate, or as often cited, the C:N ratio, influences the nitrogen cycling. In less than 4 months we could observe a change in the N dynamic of the soybean cultivation area with slight increase in δ15N and nitrification rate and significant increase in the abundance of ammonia oxidizers. Soil gross N transformation was modified in a conversion from woodland to tea plantation in an acidic oxisol, measured in lands where plantation was established after 1, 5 or 30 years; NO_3^- -N production by nitrification and N_2O increased and NO_3^- -N immobilization decreased according to the time after conversion. In addition, under the woodland, nitrification was mostly heterotrophic, contrary to both autotrophic and heterotrophic nitrification in the tea plantation soil as measured by ^{15}N trace experiment (Zhu *et al.*, 2014). Finally, nitrification in Cerrado soils might be performed by heterotrophic organisms also able to denitrify so nitrate does not accumulate (Kuenen and Robertson, 1994).

In the microcosms experiment, the pH-changed treatments had no significant difference between acetylene treated and non-acetylene microcosms, which might suggest that the observed increase in NO_3^- -N was a result of heterotrophic nitrification. Furthermore, there is evidence that the *amoA* community present in the Cerrado soils is not performing ammonia oxidation. Thaumarchaeota are often related to N metabolism in soil, but the 1.1c cluster is abundant in soil but not related to ammonia oxidation (Weber *et al.*, 2015). The higher ratio of thaumarchaeal 16S rRNA:archaeal *amoA* found in the undisturbed soil than in the soybean field is an indicative that a greater part of the archaeal community in these soils might be from 1.1c cluster.

Inhibition of nitrification

Nitrification is the main focus of this work and many others, however mineralization is the first step to be consider as it is responsible for the release of inorganic N (SCHIMEL). As fungal/bacterial abundance ratio increases in lower pH (Bárcenas-Moreno *et al.*, 2016), fungi might have higher effect on organic matter mineralization in acidic soils. The low abundance of ammonia oxidizers in Cerrado soils may be due to competition with soil fungi for ammonium or inhibition by bioactive compounds synthesized by fungi (Suzuki *et al.*, 1974).

In addition, some plants produce inhibitors of nitrification that preserve N in NH4+ form in the soil (Kölln *et al.*, 2016), named biological inhibition (BNI) promoted by plants (Subbarao *et al.*, 2015) (Subbarao *et al.*, 2009) (Zakir *et al.*, 2008). Root extracts from plants used in agriculture (*S. spontaneum*, species that forms sugarcane hybrids cultivated) or pasture (*B. humidicola*) (Kölln *et al.*, 2016) or soils cultivated with brachiarias (Fernandes *et al.*, 2011) (Subbarao *et al.*, 2009) in Cerrado biome decrease NO_3^- accumulation in soil, but less than the inhibitory effect of DCD.

Another example is the selection of specific subset of the soil bulk microbial community, which appears to be related to growth promotion and nutrition, in soybean rhizosphere (Mao *et al.*, 2011; Mendes *et al.*, 2014). Albeit possible for Cerrado native plants, this has not been demonstrated yet and neither soil solution from native Campo sujo nor soil sampled at same pH in nitrifying Craibstone station, did not inhibit AOA or AOB pure cultures. The activation of BNI synthesis depends on the exposition to a higher concentration of NH_4^+ than NO_3^- (Subbarao *et al.*, 2015), which is the case of the Campo sujo soil here studied. Despite that, the general assumption of low nitrifiers abundance in bulk soil, and the potential ability of hydrophilic BNIs to diffuse in soil, it is still possible that BNIs are only relevant in the rhizosphere and for the microbial community present in this microhabitat. However, bulk soil from Campo sujo just did not inhibit as it stimulated ammonia oxidation from organisms from the Craibstone soil in slurries. In addition, the detection of AOA *amoA* transcript increased after the 21 days in the Campo sujo, indication of activity in the AOA community despite undetectable NO_3^-.

Fe

Moreover, inhibition does not need to be biotic. For example, NO_3^- can be immobilized biotically and abiotically. The adsorption of NO_3^--N to free Fe oxide might to be

considered; especially as Fe concentration was high in the Cerrado soils sampled in this study. In temperate forest soils, abiotic immobilization of $^{15}NO_3^-$ to the DO^{15}N occurs within minutes (Dail *et al.*, 2001) and is independent of soil N status, contrary to the negative correlation observed between microbial N immobilization and soil N concentration (Johnson *et al.*, 2000). Net nitrification in subtropical acid soils was also significantly inhibited by Fe oxide addition in the form of hematite in cambisols, and AOA and AOB gene abundance decreased (Jiang *et al.*, 2015). Total Fe concentration in the Cerrado soils studied (ranged from 46 to 375 mg dm^{-3}) here were higher than the values mentioned by Jiang *et al.* (2015) for the subtropical ferralsols with high NO_3^- immobilization and low net nitrification, supporting the hypothesis that abiotic NO_3^- immobilization takes place in soils from the Cerrado. The mechanism involves reduction of nitrate to nitrite catalyzed by Fe(II) minerals in soil, that being more reactive, reacts with DOC, producing DON which would be available to heterotrophic use (Davidson *et al.*, 2003). The model proposed by Zhu *et al.* considers the increase of the abiotic adsorption of NO_3^- -N in tea plantation than in the woodland control to be related with the higher concentration of Fe oxides measured in the soil under tea plantation (Zhu *et al.*, 2014).

Yet, the low abundance of ammonia oxidizers observed in the Cerrado soils is congruent with the low values of nitrate obtained and the hypothesis of N retention in those soils. Nonetheless, the possible interference of Fe in the accurate measurement of NO_3^- concentration is debatable (Colman *et al.*, 2008; Davidson *et al.*, 2008), and different methods (Yang *et al.*, 2012) should be further tested with the Cerrado soil. Abiotic retention of nitrate should be considered, but is not the only explanation, as the observed increase nitrification in the soybean cultivation soils occurred despite the permanence of measurable levels of Fe in these soils. In this case, the Fe oxidation state in soil should be evaluated as well.

Soil texture and water contents

Another variable to be considered is microbial substrate, as microorganisms are not planktonic in soil, and are most likely protected from pH fluctuations in soil, as suggested in culture with added vermiculite (Allison and Prosser, 1993). Furthermore, clay particles and the presence of charcoal shaped the bacterial community structure, which stablished in a non-stochastic manner, as shown for the inoculation of artificial soils with different mineral composition (Ding *et al.*, 2013). Similarly, nitrification was stimulated by increasing soil

particle surface due to higher abundance and activity of both AOA and AOB in an acidic soil (Jiang *et al.*, 2011).

Nowadays, researchers are aware of the need to consider microhabitats between soil particles to understand the microbial response to disturbances in micro-scale (Vos *et al.*, 2013). Soil texture influences the size of pores, water capacity, and soil particles charge. Potentially this affects the microbial community, as found for the Cerrado conservation parks that presented higher α-diversity in sandy soils, therefore lower pore connectivity and lower competition between cells or for substrate (Carson *et al.*, 2010). Fine particles allow for a greater colonization because of greater surface area as showed for the addition of pure culture of *Nitrosomonas europaea* with ammonia-treated vermiculite (Armstrong and Prosser, 1988). Ammonia oxidation occurs preferentially at the surface of vermiculite, but not all ammonia is used by the bacteria possibly because of ammonium adsorption to the clay particles (Armstrong and Prosser, 1988).

Soil texture is directly correlated with water soil capacity, soil minerals and metals. Such that Cerrado rupestre was the driest soil sampled, and the soil with greatest composition of sand. The two conservation parks differ mainly in soil texture, varying from loamy sand (CR in PNCV) to clay (CD in PESA). Most of them were classified as sandy clay loam (PNCV: MG, SS; PESA: MG, SS, FSD). The soils in PESA have higher clay content than those in PNCV, even though there was a significant difference in soil texture within the samples in PNCV. Nevertheless, in all of them, the phyla Proteobacteria, Actinobacteria and Firmicutes were the most abundant.

Furthermore, water availability is considered one of the main drivers of the vegetation gradient in the Cerrado (Bustamante *et al.*, 2006), and of microbial distribution, which is reasonable if we consider that microbes are confined to a thin layer of water in the soil particles, and that water limits prokaryotic life in soil (Fenchel, 2012). This was observed in the metagenomes, as Campo limpo, the vegetation type with greatest soil water content, had significantly more genes annotated for motility and chemotaxis than the other soils in PNCV ($p<0.0001$). Campo limpo had also a significant higher frequency of genes annotated for nitrogen fixation, in accordance with the high abundance of sequences from the order Rhizobiales (more than 50% of bacterial sequences, $p=0.048$), mainly represented by Bradyrhizobiaceae. Even though N fixation is often correlated with symbiotic interactions, which are major for plant nutrition, in the Campo limpo, the microorganisms performing N fixation are most likely free-living.

Considering the relevance of water in Cerrado terrestrial ecosystems and the effect of rainfall on soil microbial communities (Bresolin *et al.*, 2010; Mendes *et al.*, 2012; Pinto *et al.*, 2006; Viana *et al.*, 2011), and that soil moisture may explain potential nitrification rate with the soil water content more than pH (Stempfhuber *et al.*, 2015), an experiment was designed to test the effect of soil moisture on nitrification in a Cerrado Campo sujo soil. For instance, in a Chilean semiarid soil and in the seasonally dry Californian grassland, water addition promoted a change in the community of ammonia oxidizers and increased nitrification (Bustamante *et al.*, 2012a; Placella and Firestone, 2013). Initially, Campo sujo soil was air-dried to 8% soil water content, which is a value previously described as normal during dry season in the Cerrado soils, and was also found in the Cerrado rupestre in PNCV. It was assumed that by increasing soil water content to 40% with or without previously air-drying the soil, solute transportation or cell mobility would facilitate ammonium availability for ammonia oxidizers. Furthermore, the water addition after drying the soil would lead to a birch effect (Birch, 1964) with higher N mineralization, therefore providing substrate for AOA especially known to prefer inorganic forms of N (Prosser and Nicol, 2012). However, we could not detect the expected effect of soil moisture over nitrate production. NO_3^--N was only detected after 21 days, indicating once more that the time of experiment might have been short to promote a change in the community.

As mentioned before, Cerrado has annual draught during winter and the beginning of the rainy season, and also the addition of water experimentally, promotes an increase on microbial biomass (da Silva, 2004; Nardoto and Bustamante, 2003), microbial activity and nitrification rates (da Silva, 2004), change the bacterial composition with the transition of dry season to the rainy (Bresolin *et al.*, 2010; Nardoto and Bustamante, 2003; Pinto *et al.*, 2006). Generally, low emissions of NO and N2O are associated with soils in which NH_4^+ is the dominant form of inorganic N and the pool size can be a good indicator of whether the system is open to nitrate leaking or conserves N in ammonia (Davidson *et al.*, 2000). Litterfall C:N ratio also reflects N availability and consequently predicts NO and N2O emissions from soils (Davidson *et al.*, 2000). Davidson et al (1990) suggested that in low N availability soils, nitrifying bacteria starve and low population capacity is associated with low nitrification potential (Davidson *et al.*, 1990).

Heil et al (2015) highlighted the relevance of considering the coupling between abiotic and biotic reactions (Heil *et al.*, 2015). For example, the delay between ammonia and nitrite oxidizers recovery after rewetting a dry soil can possibly allow for NO_2^- accumulation (Gelfand and Yakir, 2008), otherwise unusual as ammonia oxidation is considered the rate-

limiting step of nitrification. Consequently, higher NO_2^- concentration after the first rain leads to pulses of nitrogen trace gases emissions, as HONO and NO can be abiotically self-decomposed from NO_2^- (Su *et al.*, 2011) or directly produced by AOB (Oswald *et al.*, 2013). In the microcosm experiments, conclusions for little influence of soil moisture on nitrification were taken from the undetectable concentration of NO_x (data not shown). However, if the recovery rate of nitrite oxidizers is delayed, follow-up experiments should measure nitrite specifically and *amoA* transcripts, which was not possible with the soil used in the microcosms as RNA recovery was not efficient with the methodology used. Special attention should be payed to AOA *amoA* as NO may be an intermediate in the archaeal ammonia oxidation pathway (Stahl and de la Torre, 2012), and as studies with gases show an increase in NO emission after water addition (Pinto *et al.*, 2002), otherwise NO and N2O emission are near limit of detection and lower than the observed in Amazon sites (Verchot *et al.*, 1999).

Most likely the low NO and undetectable N2O emissions are related with the low levels of NO3- and nitrification in Cerrado soils, as well as with the high NH_4^+-N:NO_3^-N ratio found. Nonetheless, the N gases emissions are expected to increase after land use change (Weitz *et al.*, 1998). N conservation in a Namibian savanna soil was also correlated with the low availability of N for nitrification and denitrification; and the use of low amounts of fertilizers did not increase significantly the N_2O emissions when compared to the native savanna (Braker *et al.*, 2015). Nevertheless, the conversion of land use and the increased availability of C is expected to change denitrification in these soils, as low N2O emission was associated with low organic matter beside soil drainage and low nutrient levels (Castaldi *et al.*, 2006).

Increase in soil moisture is associated with increased N loss either with NO_3^- leaching or runoff after a rainfall or by emission of N gases during denitrification. Consequently, the regulation of NO or N_2O emission are genetic, ultimately, as it depends on the abundance of microbial guilds for nitrification and/or denitrification. Modular reactions characterize denitrification and can be performed by different organisms (Graf *et al.*, 2014). These might contain genes for the reduction of nitrite, nitric oxide and nitrous oxide, or just one of the above (Graf *et al.*, 2014). Therefore, the greater relative abundance of nosZ gene, especially of clade II, the greater sink capacity for N2O (Jones *et al.*, 2014). This trait is a polyphyletic characteristic, found in *Bacteria*, *Archaea*, and Fungi. Most of the organisms capable of reducing nitrate and nitrite are heterotrophic aerobic able to live in anaerobic environments. Although Cerrado soils are well-drained these organisms may be able to

denitrify in semiarid soils (McLain and Martens, 2006) (Braker *et al.*, 2015). In addition, McLain and Martens (2006) highlight the relevance of heterotrophic nitrification-denitrification in N2O emission by fungi in semiarid soils (McLain and Martens, 2006).

Cerrado vegetation cover and land use change

The Cerrado is composed by a gradient of trees/shrubs layer ranging from grasslands to forests and savannas. Both analysis of phospholipid fatty acids and 16S rRNA genes have showed that this vegetation cover influences the soil microbial composition (Araujo *et al.*, 2012; Mendes *et al.*, 2012; Viana *et al.*, 2011). However, agriculture and managed pasture for cattle breeding changed Cerrado landscape in approximately 53% (Beuchle *et al.*, 2015), with increasing alterations in floristic composition and edaphic characteristics due to fertilization, liming, and crop monoculture itself.

Soil management and monoculture crops are associated with a decrease in total and microbial N (Hernández-Hernández and López-Hernández, 2002; Peixoto *et al.*, 2010; Vinhal-Freitas *et al.*, 2012) (Bresolin *et al.*, 2010; Paula *et al.*, 2014). Land use change can alter soil sink (or source) capacity for N gas emissions, which can be produced during nitrification and denitrification. In view of the economic and ecological costs of fertilization and N losses, it is important to investigate nitrifiers in Cerrado soils to develop better soil management practices.

We showed the short-term modification on AOA and AOB abundance along a soybean culture. The change in the abundance of ammonia oxidizers was associated with the increase in pH, but in turn, the soil pH decreases the availability of Al^{+3} and other cations, and as we have described above, the presence of Fe in soil can alter its capacity of NO_3^- absorption.

Not only we can see that N dynamic changes according to several soil biotic or abiotic variables, the change in the microbial community is related with the input and output of N forms in soil. Mostly, the input of fertilizers in agriculture, aimed at a higher plant productivity, promote a shift in the microorganisms performing mineralization, nitrification and other processes, and leads to higher nitrate leaching and N gases emission. None of this is new, but clearly more changes towards a more sustainable agriculture is needed, as no one expects agriculture to stop growing, but to be more effective. Moreover, there is a debate in the use of microorganisms as indicators of soil quality (Mendes *et al.*, 2016).

Although the work in the present thesis was performed only in one type of soil in a farm land area in recovery from gravel, native soil was sampled in two other areas and showed similar patterns of low nitrate accumulation and potentially low abundance of ammonia oxidizers. Therefore, it is reasonable to assume that similar changes can take place in other areas of Cerrado. The impact of soil history on the microbial community was evaluated in the Cerrado: even after 17 years of succession from recovery of agricultural use the microbial community was still more similar to that present in the soil under a monoculture community than the one found in an adjacent native area (Rosolem *et al.*, 2003).

Considering the above, it is suggested that microbial phylogenetic and/or functional potential diversity should be considered in models. As an example, microbial stoichiometry is more and more considered in models of carbon or other nutrients cycling. For example, the lower microbial carbon use efficiency (the fraction of assimilated C used for growth rather respiration) is related with higher C:N (more recalcitrant) plant inputs (Averill *et al.*, 2014). In addition, it was suggested that microbial N:P ratios, better than those from plants, can help the assessment of nutrient limitation in terrestrial ecosystems, at least in the tropical rain forest P-limited in Costa Rica (Cleveland and Liptzin, 2007).

On the other hand, Graham *et al.* found that models on edaphic parameters were not improved by data on microbial gene abundance, but they also criticized that they might have missed environmental factors that better explain microbial community structuring. Furthermore, they suggest the inclusion of temporal dynamics in models to understand edaphic factors and microbial communities on the ecosystem functioning (Graham *et al.*, 2014).

Final considerations and new hypotheses

Metagenomic studies have been of great importance to show the potential diversity of an environment, however it has a tendency of amplifying the most abundant microorganisms in the sample, which justifies the fact that 97% of the annotated genes are bacterial. This is also related with the fact that only 2 genes were annotated as ammonia monooxygenase in the metagenomes, as qPCR analysis showed AOA and AOB abundances between 10^3 to 10^5 maximum, most likely under the threshold of capturing with the coverage used with 454 sequencing. Considering the higher number observed of AOA in the Campo sujo (10^5), and that microorganisms occupy 5% of the soils pore space, and

considering a density of 1.2 g/dm^3 for the Cerrado's soil, we find that microbial cells occupy 10^{+15} µm^3 of pore space. The average cell size is 0.6 µm^3, therefore there are around 67 millions of microorganisms in 1 g of Cerrado soil, which is accordance to previous culture-independent descriptions (Roesch *et al.*, 2007). According to this there is 1 *amoA* of *Archaea* per 10^7 cells, approximately. Comparing our findings with other metagenomes available (IDs 4477751.3-4478937.3 (Mendes *et al.*, 2014), IDs 4578924.3-4578926.3, 4577669.3-4577672.3, 4578714.3 (Souza *et al.*, 2016), project "Biodiversidade microbiana do bioma caatinga" (Lopes *et al.*, 2016), IDs 4485218.3-4485219.3, IDs 4493544.3-4493893.3 (Navarrete *et al.*, 2015)) in the MG-Rast platform there is an average of 1 copy of ammonia monooxygenase per 100 thousand sequences, independent of the sequencing methodology or the sample. Although there were samples that presented higher abundance as for example the environmental samples from the Paraguaçu river (Lopes *et al.*, 2016).

On the other hand, this is the first assessment of the N metabolism in the Cerrado with metagenomic data, and these data might help understand the impact of land use change on soil microbiota on this Brazilian savanna and consequently in the ecological processes by them produced. Although metagenomics allowed a holistic assessment of the N cycling in this study, low abundance genes are ignored and the valuation of relative abundance of processes had to be carefully discussed. The direct amplicon sequencing or the measurement of abundance of specific genes by qPCR are more advised in studies aiming at the balance of microbial community due to biotic or abiotic disturbance. Although a recent study highlighted the relevance of metagenomic and single-cell techniques to tackle the unclassified sequences obtained with amplicon due to primer bias (Eloe-Fadrosh *et al.*, 2016). In addition, when working with databases one should be aware of where to look for the genes, as we found the *amo* genes classified as membrane transporters and not in the list of genes for the subsystems of N metabolism. This is reasonable since the gene *amoA* used to quantify the abundance of ammonia oxidizers codes for the membrane-bound AMO enzyme, that takes NH_3 rather than NH_4^+ as a substrate.

The little accumulation of nitrate in the treated microcosms or in the slurries, and the absence of the inhibition effect observed in slurries and pure cultures suggests that some other mechanism occurs in this ecosystem to preserve inorganic N preferentially in the NH_3 form. It is likely that not only the presence of ammonia oxidizers is fundamental for nitrification to occur, but that the microbial community composition and diversity affects the direction in which N process occur in soil, as showed by the higher number of bands in the DGGE analysis in the Craibstone AOA community than in the Campo sujo soil. As

"narrow processes" have additive functionality (Levine *et al.*, 2011), the lower number of bands of *amoA* gene detected in the *Campo sujo* soil can be related with the lower nitrification activity. On the other hand, we could not detect the expected effect of soil moisture over NO_3^- production, indicating once more that the time of experiment might have been short to promote a change in the community.

Most likely there is a relation between abiotic and biotic conditions that limits the microbial community to low abundance of autotropic ammonia oxidizers possibly towards an ecosystem N conservation. Figure 21 repeats the basic N cycle that occurs in terrestrial ecosystems (annanmox was excluded) presented in Figure 1 and includes now the influences evaluated in this work and/or considered in the discussion.

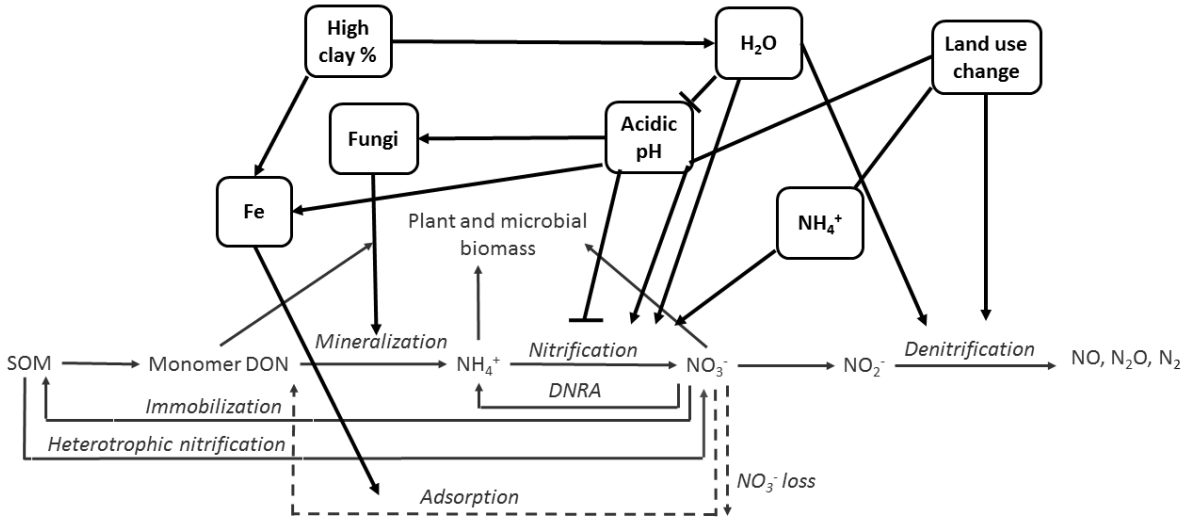


Figure 22. Interaction between abiotic and biotic factors and their effect on the N cycle processes

Others have showed environmental factors that affect ammonia oxidation (Erguder *et al.*, 2009). In this study we observed that tillage, fertilization, liming, and soybean monoculture altered soil pH, moisture, and inorganic N contents, all of which can influence the abundance and diversity of microbial communities and their functional potential, thereby influencing the production of NO_3^-, NO_2^-, NO, and N_2O (Mao *et al.*, 2011). The change after fertilization and liming illustrates the prevalence of determinate species in nutrient utilization, and highlights the shift in the community related with the nutrient dynamic, loss and conservation.

Moreover, microbial ecology research needs to consider microbial cells physiology as different organisms have knowingly diverse growth rates and to not observe increase in abundance or growth of an organism does not mean necessarily that this one is not active, but that something is limiting its growth; limitation that can be either biotic or abiotic as exposed above. Allison and Prosser (1993) suggested that even though cells can be actively oxidizing ammonia at low pH, the energy produced is enough to maintain cell but not for growth (Allison and Prosser, 1993). In this case, metatranscriptomics helps to better determine who is active in certain conditions (Prosser, 2015) regardless of the change in gene abundance (as increase in gene abundance is a presumptive measure of growth in incubation assays). However, RNA extraction from soil is not as simple as for DNA and in this work it was more successful after soil incubations, where we could successfully observe an AOA *amoA* transcripts in the Campo sujo-only slurry incubation after 21 days.

Some other process of NO_3^- use by the microbiota should be considered in further studies, i.e. competition for NH_4^+ by plants or heterotrophic microbes, as described in Californian grasslands (Jackson *et al.*, 1989); dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) (Chen *et al.*, 2015; Cole and Brown, 1980); abiotic NO_3^- immobilization according to the ferrous wheel hypothesis (Jiang *et al.*, 2015), or the clay fixation of NH_4^+.

The competition for NH_3 between plant roots, nitrifying and heterotrophic bacteria has been reviewed before (Verhagen *et al.*, 1994). Verhagen *et al.* showed that heterotrophic and plant roots win the competition for ammonium, in this order, against nitrifiers, and they also could not find a nitrification inhibition by plants allelochemicals. However, the works considered by them have compared heterotrophic organisms with *N. europaea*. New studies should consider the competition for NH_3 between hetetrophic with AOA and AOB.

This thesis focused on the understanding of autotrophic ammonia oxidation by *Archaea* and *Bacteria* in the Cerrado soil, but the results obtained, along with the literature suggest that future work should expand on the heterotrophic nitrification in these soils and specifically with regard to fungal community. The use of specific inhibitors for bacteria or fungi show the capacity of fungi to nitrify NH_4^+ and organic N in grassland soils (Laughlin *et al.*, 2008). Zhu et al. also suggest that the higher NO_3^- immobilization rate in the soils under the woodland than in the tea plantation was an efficient way of conservation of produced NO_3^- by the heterotrophic nitrification (Zhu *et al.*, 2014).

This work focused on the microbial perspective of the natural conservation of N in the Cerrado soils. Our look is misleading, more preoccupied with the systems that increase

N loss than understanding how some systems, and their microbiota, maintain low N losses. The input of fertilizers in lower concentration, the maintenance of high C:N ratio with addition of organic matter rich in C or the non-continuous supply of nutrients (i.e. the use of biochar), which seems to keep a dynamic between plants and microorganisms are some solutions to be addressed to lower nutrient loss. But also, the change of land use leads to a decrease in soil microbial diversity; in turn, loss of functional diversity has been associated with the decrease in ecosystem multifunctionality (Bradford *et al.*, 2014), potentially reflecting on the provision of ecological services. So greater aboveground diversity might allow similar increase in the belowground and consequently facilitate the sustainability of soil functions.

Paralleling what the philosopher Edgar Morin suggests for complex systems, the study of soil needs to distinguish the components that compose the soil and to consider that the all is formed by smaller parts, but these, in turn, interact with each other in a way that the sum of parts is smaller than the all. The tendency is to have more multidisciplinary studies linking soil physics, plant physiology and genetics, soil microbiology, biostatistics, network modelling to understand soil's behavior face to natural or anthropogenic disturbances.

Capítulo 6 – Conclusões e perspectivas

✓ Baixas taxas de nitrificação líquida nos solos do Cerrado

✓ Alta abundância de genes relative à oxidação de amônia

✓ Presença de oxidantes de amônia em solos do Cerrado, dos Domínios *Archaea* e *Bacteria*, no entanto, em baixa abundância quando comparado a outros solos

✓ AOA são mais abundantes que AOB, tal como esperado devido ao baixo pH típico dos solos de Cerrado

✓ O cultivo da soja (manejo, fertilização, calagem e a monocultura) alteraram a abundância de AOA e AOB em um curto período de tempo (134 dias)

✓ AOA e AOB podem estar dormentes ou desenvolvendo outras funções nos solos do Cerrado

✓ A nitrificação não é limitada por pH, água ou inibidores biológicos

✓ O perfil de DGGE indica uma composição de baixo número de bandas de AOA, sugerindo uma baixa diversidade desse grupo nos solos de Cerrado

✓ Em termos de processos de baixo espectro, como aqueles relativos ao ciclo do nitrogênio, a diversidade e a estrutura da comunidade microbiana são importantes fatores para o funcionamento do ciclo

Considerando o que foi relatado nos trabalhos desta tese, algumas perspectivas para trabalhos futuros são enumeradas abaixo:

- A comunidade fúngica deveria ser considerada em futuras análises da limitação do crescimento de oxidantes de amônia
- A modificação na dinâmica de N dirigida à conservação de NH_4^+ pode ser avaliada considerando também a taxa bruta de nitrificação pelo método de diluição de $^{15}NO_3^-$
- Devido à maior razão entre NH_4^+ e NO_3^- nos solos do Cerrado, um estudo futuro deve avaliar a comunidade de organismos que realizam redução dissimilatória de nitrato a amônia
- O estudo do fluxo de N nos compartimentos do solo e a microbiota associada à assimilação de N pode ser acompanhada pelo isótopo 15N

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