UNIVERSIDADE DE SÃO PAULO
CENTRO DE ENERGIA NUCLEAR NA AGRICULTURA

DANIEL RENATO LAMMEL

Soil microbiota related to carbon, nitrogen and greenhouse gas cycles across different land uses in Southwestern Amazonia

Piracicaba
2011

DANIEL RENATO LAMMEL

Soil microbiota related to carbon, nitrogen and greenhouse gas cycles across different land uses in Southwestern Amazonia

Tese apresentada ao Centro de Energia Nuclear na Agricultura da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

Áreas de Concentração: Química na Agricultura e no Ambiente (CENA/USP); Microbiologia (UMASS)

Orientador: Prof. Dr. Carlos Clemente Cerri
Co-orientador: Prof. Dr. Klaus Nüsslein (UMASS)

Piracicaba
2011

Dados Internacionais de Catalogação na Publicação (CIP)
Seção Técnica de Biblioteca - CENA/USP

Lammel, Daniel Renato

Soil microbiota related to the carbon, nitrogen and greenhouse gas cycles across different land uses in Southwestern Amazonia / Daniel Renato Lammel; orientador Carlos Clemente Cerri, co-orientador Klaus Nüsslein. - - Piracicaba, 2011.

151 f.: il.

Tese (Doutorado – Programa de Pós-Graduação em Ciências. Área de Concentração: Química na Agricultura e no Ambiente) – Centro de Energia Nuclear na Agricultura da Universidade de São Paulo.

1. Cerrado 2. Ciclos biogeoquímicos 3. Desenvolvimento sustentável – Amazônia 4. Ecologia microbiana 5. Efeito estufa 6. Microbiologia do solo 7. Mudança climática 8. Reação em cadeia da polimerase 9. Uso da terra (Economia) I. Título

CDU 550.47 : 579.26
CDD 574.5222

To all people that look for a fair
and sustainable world,
I offer this work.

Acknowledgments

To God, for everything. To my family, for unconditional support and love.

To Prof. Carlos Clement Cerri, for providing all of the resources so that this work could be completed. I appreciate the opportunity to work in his research group on the project titled “Agricultural Impact on Southwestern Amazon”. I am really thankful for all the scientific, bureaucratic, and personal support that allowed me to complete this work.

To Prof. Siu Mui Tsai, for the first invitation to work on this project. I really appreciate all the support, especially in the project development, first field survey, mesocosm experiments, and qualification exam. I am really thankful; your support was essential to this work.

To Prof. Klaus Nüsslein, for unconditional support at UMass. I appreciate the opportunity to work in your research group and to have been a Ph.D. student at UMass. I am really thankful for all the scientific, bureaucratic, and personal support that allowed me to have extraordinary life and learning experiences and to greatly improve my work.

To Prof. Adriana Pinheiro Martinelli, coordinator of the CENA-USP Graduate Program, for all the support during the doctorate. Your support was essential to studying abroad and for this thesis to be finished. I am really grateful for your attention and support. I am also grateful for all the CPG staff for their support.

To Brigitte J. Feigl and Carlos Eduardo P. Cerri for important discussions about the GHG, C, N, and biomass. I am really thankful for the support in planning the experiments and analyzing the data.

To Dean John Mullin, Nigar Khan, Frank Hugus, Jack Ahern, John Lopes, Maryanne Wells and all the University of Massachusetts Amherst staff that gave support to the Ph.D. at UMASS.

To Prof. Klaus Butterbach-Bahl, for allowing me to the train in Germany. To Jörg Schnitzler for allowing me to work in his laboratory and, together with Sandrine Louis, for advising me in my work. For Nicolas and Ralf for important discussions. And to all for important suggestions in the analysis. To all the IMK-IFU staff, especially to Regina Wiegel for support in the lab analysis.

To Marilia R.G. Henyei for support with the review of the thesis format. I really appreciate all the attention in such short notice. You were very efficient and kind and I am really thankful.

To Adelmo Zuanazzi, José Alves da Silva, Vitor Poltronieri, José Puppin, Bruno Puppin e Jader Bergamasco for allowing the soil survey in their farms. I really thank you for all the support in the farms.

To all colleagues and friends that helped in this work. Without your support, this work hardly would be done. To my friend Thamaturgo Castro for unconditional support in Sinop; thank you so much for help finding the most adequate study areas and for all the logistic support. To Wagner Piccinini for tremendous help in the soil survey, especially the first one in 2009, that resulted in almost 700 kg of soil and 5.000 km driving. I am also grateful for the help of Ezio Nalin, Marcos Siqueira, Gregori Ferrão, Diana Signor, João Luis Carvalho, Prof. Carlos Cerri and Luiz Cláudio during the first survey. I am also grateful for Wagner, Lucas, Acácio and Castro for survey the soil for chapter 6. Thank you for my friends Clovis Borges and Angelica Jaconi for helping me with the lab work and for always supporting me with the Ph.D. Thanks Lucas Mendes for support with T-RFLP analysis. Thank you Marcos Siqueira and João Carvalho for the important discussions; Marina Miyauchi for helping with the thesis figures; Carol Lisboa and Richard, Foreman for the support in Germany. To Adriana Inviti and Rafaela Neroni for help in the introduction. To the friend Marcelo Schneider for English support. I am also grateful for the help of all the other colleague and friends, Adriana, Alessandra, André, Arlete, Bruna, Chico Fujita,Cindy, Ciniro, Débora, Denise, Dennis, Felipe, Gabor, Leidi, Magnus Deon, Marcelo Galdos, Karine, Maisa, Marilia, Mylenne, Rafael Bolinha, Rafael Vasconcelos e Stoécio.

To the colleagues and friends at UMASS George, Kyunghwa, Burcu, Deepti, Cristian, Chloe, Jen, Jesus, Roberto, Hector, Kewei, Kahlil, my roommate Stefan, family Ford, family Brose; and especially to Carlton, Caryl, George, Jill and Theresa for helping with the English.

I am thankful for the technical support of Lillian, Dagmar, Sandra, Ralf, Chiquinho, Elias, Fábio, Denise, Mara, Zezinho, João, Elisangela, and for the professors Elke Cardoso, Fernando Andreote, Marcio Lambais and Carlos Tadeu.

To my grandfathers, who first introduced me to agriculture and got me exited to work in this area. *In memoriam* of my grandfather Rudolf G. Zeidler, who first introduced me to the N cycle, showed me the “azoto” in the roots of a crotalaria species between the rows of the coffee plantation on his farm. To my grandfather Erno Lammel, who first introduced me to the Cerrado and to his concerns about preserving the native vegetation and animals, in combination with soybean and cattle production on his farm. To the Boy Scouts movement, that first introduced me to the environmental responsibility and my love for the forest, with hiking and camping in the Atlantic Rainforest. To my hometown Curitiba, that with its educational and environmental policies, allowed me to grow thinking in terms of sustainability.

The color blue in the last picture was chosen to support a stronger United Nation, the only way to build a more sustainable and fair world. The pink color was used in support to the world campaign against Cancer.

This thesis could not be finished without support of God, my family, my friends, and all the persons above, and I am really thankful to all!

To USP and FAPESP, by means of projects 2007/07570-6 and 2005/59012-1 for financial support. To UMASS Amherst for support the PhD in USA.

“*You must teach your children that the ground beneath their feet is the ashes of our grandfathers. So that they will respect the land tell your children that the earth is rich with the lives of our kin. Teach your children what we have taught our children that the earth is our mother. Whatever befalls the earth befalls the sons of the earth. Man did not weave the web of life, he is merely a strand in it. Whatever he does to the web, he does to himself.***“**

Chief Si'ahl, 1854

RESUMO

LAMMEL, D. R. **Soil microbiota related to carbon, nitrogen and greenhouse gas cycles across different land uses in Southwestern Amazonia**. 2011. 151 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2011.

Sustentabilidade é um dos maiores objetivos da humanidade no novo milênio. Uma demanda crescente por produtos agrícolas tem estimulado a expansão agrícola no Brasil, especialmente no Sudoeste da Amazônia, nos biomas Cerrado e Amazônia. Um melhor entendimento dos ciclos biogeoquímicos e suas influências em sistemas naturais e agrícolas é chave para se alcançar sustentabilidade ambiental e aumentar eficiência agrícola. Esses ciclos biogeoquímicos são guiados por microrganismos, e o objetivo dessa tese foi correlacionar abundância de grupos funcionais de microrganismos com carbono, nitrogênio e gases de efeito estufa (GEE) em resposta a mudança do uso da terra em solos do sudoeste da Amazônia. Esse trabalho foi realizado na Universidade de São Paulo e na Universidade de Massachusetts Amherst enquanto o doutorando esteve matriculado nas duas universidades. A tese é composta de cinco estudos. O primeiro estudo mostra que a mudança no uso da terra de Cerrado e floresta para agricultura (soja, *Glycine max* (L. Merrill), em sucessão com outros cultivos) ou pastagem (*Brachiaria brizantha* (Hochst. ex A. Rich.) não reduz diversidade microbiana, mas muda sua estrutura. O segundo estudo descreve as alterações nos estoques de C, N, parâmetros químicos e microbiológicos da conversão de Cerrado para agricultura e pastagem. No terceiro e no quarto estudos, microcosmos foram usados para avaliar a influência de soja e braquiária na microbiota dos solos. Genes chaves dos processos biogeoquímicos (*amo*A, *nir*K, *nir*S, *nor*B, *nos*Z, *mcr*A, e *pmo*A) foram quantificados e correlacionados com C, N e GEE. No quinto estudo, coletas *in situ* de solo e gases foram usadss para caracterizar a mudança do uso da terra de floresta para pastagem (braquiária, 25 anos) e para agricultura (soja, segundo ano, e soja, 25 anos, em sucessão com outras culturas). Correlações entre genes e processos foram encontradas, indicando que abundância gênica fornece importantes informações para o entendimento dos ciclos biogeoquímicos. Mudança no uso da terra como um todo, mais do que a mudança de vegetação, promove as alterações na abundância gênica e processos do solo. Durante o período de coleta, floresta exibiu maior atividade microbiana,

resultando em maior disponibilidade de nitrato e emissão de N_2O. Esses processos correlacionam com maior abundância dos genes relacionados aos processos. Quantidades de nitrato e N_2O foram menores em agricultura e pastagem. As emissões de CO_2 foram maiores na área de soja de segundo ano. Os solos de floresta e soja de segundo ano se mostraram como drenos de metano, enquanto que a pastagem foi uma fonte de emissão. Os resultados validam o uso de abundância gênica como uma técnica valiosa para um melhor entendimento dos ciclos do C, N e GEE. Os genes *nir*K, *nos*Z, e 16S rRNA apresentaram as melhores correlações com os processos. Uma análise temporal e espacial mais abrangente é necessária para generalizações sobre a dinâmica dos processos na região estudada. Pela primeira vez abundância gênica foi usada para integrar os ciclos do C, N e GEE, colaborando para um melhor entendimento dos processos relacionados à mudança no uso da terra no sudoeste da Amazônia.

Palavras-chave: Mudança no uso da terra. Amazônia. Cerrado. Soja. Pastagem. Gases de efeito estufa. Ciclos biogeoquímicos. Abundância gênica. qPCR.

ABSTRACT

LAMMEL, D. R. **Soil microbiota related to carbon, nitrogen and greenhouse gas cycles across different land uses in Southwestern Amazonia**. 2011. 151 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2011.

Sustainability is one of the biggest goals of humankind in the new millennium. An increasing global demand on agricultural products stimulates agricultural expansion in Brazil, especially in the Southwestern Amazon, namely in the Cerrado and Amazon biomes. A better understanding of biogeochemical cycles and their influence on natural and agricultural systems is key to achieve environmental sustainability and improve agricultural efficiency. These biogeochemical cycles are driven by microbes, and the aim of this thesis was to correlate microbial functional group abundances with differences in carbon, nitrogen, and greenhouse gas cycles in response to land use changes in Southwestern Amazon soils. This work was performed at the University of São Paulo, Brazil, and at the University of Massachusetts Amherst, USA, while the candidate was enrolled in Ph.D. programs at both universities. The thesis is composed of five studies. The first study shows that land use change from Cerrado and forest to agriculture (soybean, *Glycine max* (L. Merrill), in succession with other crops) or pasture (*Brachiaria brizantha* (Hochst. ex A. Rich.) did not reduce soil microbial diversity but changed microbial structure. The second study, a physico-chemical background for this land use conversion, describes the alteration of C and N stocks, soil chemical parameters, and microbiological parameters such as biomass, biological C stocks, and changes in the abundance of prokaryotes and fungi. In the third and fourth studies microcosm experiments depict how the agricultural change to soybean and *Brachiaria* alter the original microbial structure found in forest or cerrado. These studies focused on abundances of key biogeochemical genes (*amo*A, *nir*K, *nir*S, *nor*B, *nos*Z, *mcr*A, and *pmo*A) and correlated gene copy abundances with C, N, and GHG measurements. In the fifth study, *in situ* soil surveys and GHG samplings were used to characterize the changes from forest to pasture (*B. brizantha*, 25 years) or soybean crop system (for 2 years or 25 years in succession). We found correlations between genes and processes, indicating that gene abundances provide important microbial information for the understanding of the targeted biogeochemical cycles. Land use, rather than

plant species, promotes alterations in microbial gene abundances and processes. During the survey period, forest exhibited higher microbial activity, resulting in higher nitrate availability and N_2O emissions. These processes were correlated with higher abundances of process related genes. Nitrate and N_2O emissions were lower in agricultural and pasture soils. CO_2 emission was higher in the two-year-old soybean plot. The forest and two-year-old soybean plots acted as a sink for CH_4, while the pasture plots represented a source of it. The results validated the use of gene abundance determination as a valuable tool to better understand C, N, and GHG processes. The genes *nir*K, *nos*Z, and 16S rRNA presented the best correlations with the processes. A larger temporal and spatial analysis is needed to infer statements on the processes' dynamics due to land use change. For the first time gene abundance measurements were used to integrate the C, N and GHG cycles, giving insights into land use changes in Southwestern Amazon.

Keywords: Agricultural land use change. Amazonia. Cerrado. Soybean. Pasture. Greenhouse gas. Biogeochemical cycles. Gene abundance. qPCR.

ZUSAMMENFASSUNG

LAMMEL, D. R. **Bodenmikroorganismen Im Zusammenhang Mit Kohlenstoff-, Stickstoff- Und Treibhausgas-Zyklen In Verschiedenen Landnutzungen Im Südwestlichen Amazonien**. 2011. 151 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2011.

Nachhaltigkeit ist eines der größten Ziele der Menschheit im neuen Jahrtausend. Eine globale Nachfrage für landwirtschaftliche Erzeugnisse fördert landwirtschaftliche Expansion in Brasilien, vor allem im Südwesten von Amazonien, nämlich die biome Cerrado und Amazonas. Ein besseres Verständnis der biogeochemischen Kreisläufe und deren Einfluss auf natürliche und landwirtschaftliche Systeme ist der Schlüssel dafür die ökologische Nachhaltigkeit zu erreichen und die landwirtschaftliche Effizienz zu verbessern. Bioeochemischen Kreisläufe sind durch Mikroben angetrieben. Das Ziel dieser Arbeit war es deshalb die Häufigkeiten funktioneller mikrobieller Gruppen mit den Unterschieden in Kohlenstoff-, Stickstoff-, und Treibhausgas-Zyklen zu korrelieren als Antwort auf die geänderte Flächennutzung in den Böden im südwestlichen Amazonien. Diese Arbeit wurde an der Universität von São Paulo, Brasilien durchgeführt, und an der University of Massachusetts Amherst, USA, während der Kandidat an beiden Universitäten in die jeweiligen Ph.D.-Programme eingeschrieben war. Diese Arbeit besteht aus fünf Studien. In der ersten Studie haben wir gezeigt, dass Landnutzungsänderungen von Cerrado oder von Wald zur Landwirtschaft (Glycine max (L. Merrill), Soja, nacheinander mit anderen Kulturen) oder zur Weide (Brachiaria brizantha (Hochst. ex A. Rich.) die mikrobielle Vielfalt nicht reduzieren, sondern die mikrobielle Struktur verändern. Die zweite Studie, eine physikalisch-chemische Hintergrundanalyse für diesen Landnutzungswandel, beschreibt die Veränderung der C-und N Gehalte, der bodenchemischen und mikrobiologischen Parameter wie Biomasse, biologische Kohlenstoffanteile und Veränderungen in den Häufigkeiten von Prokaryoten und Pilzen. In der dritten und vierten Studien zeigen Experimente mit Mikrokosmen wie sich durch die Veränderung der Landwirtschaft zu Soja-und Brachiariaanbau die ursprüngliche mikrobielle Struktur von Wald oder von der Cerrado verändert hat. Diese beiden Studien konzentrieren sich auf die Häufigkeiten der wichtigsten biogeochemischen Gene (*amoA, nirK, nirS, norb, nosZ, mcrA* und *pmoA*) und korrelieren die Häufigkeiten von Genkopien mit C, N, und THG-Messungen. In der

fünften Studie werden *in situ* Bodenuntersuchungen und THG-Proben verwendet, um die Änderungen vom Wald zur landwirtschaftlichen Nutzung als Weide (*B. brizantha*, 25 Jahre) oder für den Sojabohnenanbau (für 2 Jahre oder 25 Jahre in Folge) charakterisieren zu können. Wir fanden eine Korrelation zwischen Genen und Prozessen, was darauf hinweist, dass Genhäufigkeiten wichtige mikrobielle Informationen für das Verständnis bestimmter biogeochemischer Kreisläufe produziert. Landnutzung, anstatt Pflanzenarten, fördert Veränderungen in mikrobiellen Genhäufigkeiten und Prozessen. Während des Erhebungszeitraums zeigte der Wald höhere mikrobielle Aktivität, was zur höheren Nitratverfügbarkeit und zu höheren N_2O-Emissionen führte. Diese Prozesse waren mit höheren Häufigkeiten von Genen verbunden, die metabolische Aktivitäten regeln. Nitrat-und N_2O-Emissionen waren niedriger von landwirtschaftlich genutzten Böden und von Weidelandnutzung. Im zweijährigen Sojaanbau waren die CO_2-Emissionen höher. Der Waldboden und Böden die dem zweijährigen Sojaanbau dienten, verhielten sich als CH_4 aufnehmende Systems, während die Weideböden vermehrt Methan produzierten. Unsere Ergebnisse bestätigten die Verwendung von Genhäufigkeiten als ein wertvolles Instrument, um Genhäufigkeiten in C, N, und THG-Prozessen besser zu verstehen. Die stärksten Korrelationen mit diesen Prozessen präsentieren die gene *nirK*, *nosZ* und 16S *rRNA*. Eine größere zeitliche und räumliche Analyse ist erforderlich um das dynamische Verhalten dieser Prozesse als Folge von Landnutzungsänderungen erklären zu können. Zum ersten Mal wurden hier Messungen von Genhäufigkeiten verwendet, um die C, N und THG-Zyklen zu integrieren – was ein neues Verständnis der Landnutzungsänderungen im südwestlichen Amazonien erlaubt.

Stichworte: Landnutzungsänderungen. Amazonia. Cerrado. Sojaanbau. Weide. Treibhausgas. Biogeochemischen Kreisläufe. Genhäufigkeiten. qPCR.

ABBREVIATIONS

16S Arc	Archeal gene 16S rRNA
16S Bac	Bacterial gene 16S rRNA
Brachiaria	*Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf.; synonymous *Urochloa brizantha* (Hochst. ex A. Rich.) R.D. Webster
BDL	Bellow detection limit
d.s.	Dry soil
GHG	Greenhouse gases
GGE	Greenhouse gases emission
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Soy	Soybean, *Glycine max* (L.) Merrill
T-RFLP	Terminal Restriction Length Polymorphism
UMASS	University of Massachusetts, Amherst, MA
USP	Universidade de São Paulo
CENA	Centro de Energia Nuclear na Agricultura, Piracicaba, SP

SUMMARY

1 INTRODUÇÃO

O maior desafio do novo milênio é sustentabilidade. No último século a humanidade experienciou um grande crescimento populacional e de tecnologia, mas nos dias atuais esta usando recursos naturais em uma taxa quatro vezes maior do que o planeta pode suportar. Ao mesmo tempo, em algumas regiões fome e pobreza ainda são realidades (PIMENTEL et al., 1999; CAPRA; HENDERSON, 2009).

A humanidade enfrenta um grande desafio em como usar racionalmente os recursos naturais e ao mesmo tempo promover igualdade e qualidade de vida ao redor do globo. Sustentabilidade é uma palavra chave para todas as atividades humanas, visando viabilidade econômica, qualidade ambiental e justiça social.

O primeiro esforço global para sustentabilidade foi em relação ao aquecimento global. Atmosfera não tem fronteiras e apenas uma cooperação global pode adequadamente enfrentar o problema. O impacto do aquecimento global no meio ambiente e as conseqüências para a vida humana aumentaram as preocupações da comunidade científica, política e da população em geral. Essa preocupação surge dos fortes indicativos que o aquecimento global tem contribuído para aumentar recentes desastres ambientais, como tornados, secas, enchentes, e recentes publicações indicam os impactos negativos do aquecimento global para os ecossistemas naturais e vida humana (IPCC, 2007; KERR, 2005; MARENGO, 2006).

Devido à importância desses fatos, as Nações Unidas criaram o Painel Intergovernamental de Mudanças Climáticas (IPCC) para conectar a comunidade cientifica e reportar o estado do conhecimento sobre aquecimento global. Em relatórios recentes, foram apresentados dados indicando a grande probabilidade que o aquecimento global, e conseqüente desastres naturais, podem estar sendo potencializado pelas atividades humanas, fazendo-se necessário um esforço global para mitigação (IPCC, 2007).

O maior fator antropogênico causando aquecimento global é a emissão de gases de efeito estufa (GEE), principalmente CO_2, CH_4 e N_2O. A emissão desses gases no Brasil é causada especialmente pela mudança no uso da terra, de vegetação nativa para agricultura e pastagem (BRASIL, 2004).

No entanto, no Brasil o agronegócio apresenta relevante importância sócio-econômica, representando cerca de 30% do produto interno bruto (PIB) e é um setor

estratégico para geração de empregos (CEPEA, 2007). O Brasil é considerado o “celeiro do mundo”, com potencial para se tornar o maior produtor do mundo em 2017, especialmente por causa da expansão agrícola, tornando o Sudoeste da Amazônia a maior fronteira agrícola do mundo. Este desenvolvimento cria um paradoxo, pois apesar da mudança no uso da terra ser a maior fonte de emissão de gases de efeito estufa no Brasil, ao mesmo tempo apresenta essencial importância sócio econômica para o país. Sendo assim, se faz necessário um estudo detalhado para melhor compreender o fenômeno. Esse conhecimento é fundamental para contribuir na mitigação dos efeitos negativos e assessorar na sustentabilidade dessas atividades.

Enquanto na mudança do uso da terra a emissão de GEE esta principalmente relacionada ao desmatamento e queima, na agricultura e pecuária está relacionada a microrganismos, que produzem os três principais GEE, CO_2, CH_4 e N_2O (IPCC, 2007).

Esses mesmos organismos fazem parte dos ciclos biogeoquímicos do Carbono e Nitrogênio. Ambos os ciclos apresentam tremenda importância a vida. Eles estão relacionados à manutenção de fluxo de energia e reciclagem de matéria nos ambientes naturais. Além disso, um melhor conhecimento desses ciclos pode otimizar processos agrícolas, como o caso da fixação biológica de nitrogênio, que pode reduzir uso de fertilizantes nitrogenados, ou o sequestro de carbono, que reduz CO_2 da atmosfera e pode aumentar a formação de substancias húmicas e, conseqüente, fertilidade do solo (SIQUEIRA; MOREIRA, 2006; BAYER et al., 1999).

Maioria dos organismos envolvidos nesses ciclos não pode ser cultivada por métodos convencionais, fazendo-se necessário o uso de técnicas de biologia molecular, como análise da estrutura microbiana por T-RFLP, ou quantificação de genes relacionados aos processos por PCR quantitativa (VAN ELSAS; BOERSMA, 2011). Usando essas técnicas, o papel funcional das populações microbianas nos processos de transformação do solo nos diferentes usos da terra na fronteira de expansão agrícola podem ser melhor entendidos (CENCIANI et al., 2011).

É importante enfatizar que os ecossistemas Amazônicos são sensíveis ao uso da terra (FUJISAKA et al., 1998; MARENGO, 2006; CENCIANI et al., 2011). A vegetação nativa é importante para a resiliência dos ecossistemas e um manejo inadequado pode causar perturbações severas, como degradação de pastagens e desertificação. Além disso, essa região é conhecida como um dos maiores centros de biodiversidade do mundo (MARENGO, 2006) e o presente estudo apresenta uma avaliação do impacto dos diferentes usos da terra nas pouco estudadas comunidades microbianas desses solos.

Ao mesmo tempo em que essa região apresenta todos esses aspectos ecológicos, é uma importante fronteira agrícola, necessária para abastecer a crescente demanda mundial por produtos agrícolas. Ao mesmo tempo, também apresenta grande importância ao país, fornecendo crescimento econômico e colaborando para o desenvolvimento social da população brasileira.

O foco dessa tese foi estudar como os processos microbianos relacionados com os ciclos do C e N respondem a mudança no uso da terra no Sudoeste da Amazônia.

1.1 Hipóteses: A mudança no uso da terra de ambiente natural para agricultura e pecuária resulta em mudanças químicas, físicas e biológicas que causam alterações nos ciclos do C, N e gases de efeito estufa. A abundância de grupos microbianos, acessada por qPCR, pode ser um bom indicador dessas mudanças e da emissão de gases de efeito estufa.

1.2 Objetivo geral: Analisar a abundância de grupos microbianos, examinando genes relacionados aos ciclos do C, N e gases de efeito estufa em diferentes usos da terra no Sudoeste da Amazônia e estabelecendo relações com os ciclos biogeoquímicos.

1.2.1 **Objetivos específicos**:

a) Caracterizar como as comunidades microbianas são alteradas com a mudança do uso da terra de Floresta e Cerrado para agricultura e pastagem, por T-RFLP (16S rRNA) e qPCR, correlacionando com atributos do solo.

b) Avaliar a mudança no uso da terra de Cerrado para agricultura e pastagem, e o impacto nos estoques de C, N, e nos atributos microbianos, biomassa microbiana e qPCR do gene 16S rRNA *Bacteria*, *Archaea* e 18S rRNA *Fungi*.

c) Testar o efeito de planta de soja e braquiária em mesocosmos, avaliando o fluxo de gases de efeito estufa (CO_2, CH_4, N_2O) e correlacionando com atributos químicos de solo e qPCR de genes envolvidos nos processos (*nor*B, *nos*Z, *mcr*A and *pmo*A).

d) Avaliar o fluxo de gases de efeito estufa (CO_2, CH_4, N_2O) e correlacionar com atributos químicos de solo e qPCR de genes envolvidos nos processos (*amo*A, *nir*K, *nir*S, *nor*B, *nos*Z, *mcr*A and *pmo*A) em amostras de solo coletadas *in situ* em Floresta Amazônica e em áreas convertidas a pastagem e soja.

e) Procurar e discutir correlações entre abundância gênica, parâmetros do solo e análises de C, N e gases de efeito estufa.

2 INTRODUCTION

The main challenge of the new millennium is sustainability. In the last century humankind experienced a high growth in population and technology, but nowadays is using natural resources at a rate four times higher than the Earth can support. Also, while some countries experience high quality of life, inequality in the world is high, and in some regions hunger and poverty are still realities (PIMENTEL et al., 1999; CAPRA; HENDERSON, 2009).

Humankind faces a big challenge in how to wisely use natural resources and at the same time promote equality and life quality around the globe. Sustainability is a key word for all human activities, promoting economic viability, environmental quality and social fairness.

The first global effort to support sustainability was in relation to global warming. Atmosphere does not have borders, and only global cooperation can adequately tackle the problem. The impact of global warming on the environment and its consequences for human life increased the concern of the scientific community, the political community, and the population in general. These concerns are due to strong indications that global warming enhanced recent environmental disasters, such as tornadoes, droughts, and flooding, and recent publications indicate the negative impact of global warming on natural ecosystems and human life (IPCC, 2007; KERR, 2005; MARENGO, 2006).

Due to the importance of these facts, the United Nations created the Intergovernmental Panel on Climate Change (IPCC) to connect the scientific community and to report the state of knowledge of global warming. In recent reports, data were presented indicating the great probability that global warming, and consequently natural disasters, may be potentialized by anthropogenic activity, making it necessary that the mitigation of this problem becomes a global effort (IPCC, 2007).

The main anthropogenic factors causing global warming are greenhouse gas emissions (GGE), mainly CO_2, CH_4 and N_2O. The emission of these gases in Brazil is caused especially by land use change from native vegetation to agriculture and cattle raising (BRASIL, 2004).

However, in Brazil agribusiness is of high socio-economic importance representing approximately 30% of the Brazilian gross domestic product (GDP), and is a strategic sector for the generation of employment (CEPEA, 2007). Brazil is now considered the “world’s largest barn” with the potential to be the world’s largest food producer by 2017, due especially to agricultural expansion, turning Southwest Amazonia into the largest agricultural frontier in the world. These developments create a paradox for land use in Brazil, namely that land use change, i.e., agriculture and cattle raising, are the drivers of GGE in Brazil while at the same time they are essential to the socio-economic wellbeing of the country. Therefore, a detailed study is necessary to better understand this phenomenon. Findings from such a study may contribute to the mitigation of negative effects on the environment, and assist the sustainability of these activities.

While in land use change the emission of GGE is mainly related to deforestation and burning operations, in agriculture and cattle raising GGE is related to microorganisms, that produce gases of high impact in the greenhouse effect, CH_4 and N_2O (IPCC, 2007).

These same organisms are related to the carbon and nitrogen biogeochemical cycles. Both cycles present tremendous importance for life. They are related to the maintenance of energy flow and matter recycling in natural environments. Also, a better knowledge of these cycles can improve agricultural process, as the case of biological nitrogen fixation, which reduces the need for fertilizers, or the carbon sequestration, which reduces CO_2 from atmosphere and may improve humic substances formation and, consequently, increasing soil fertility (SIQUEIRA; MOREIRA, 2006; BAYER et al., 1999).

Most of the organisms involved in these cycles have not yet been cultivated by conventional methods, making the use of biochemical and culture independent molecular methods necessary, such as the study of microbial structure by T-RFLP and the analysis and quantification of genes related to these processes by real time PCR (VAN ELSAS; BOERSMA, 2011). Using these techniques, the functional role of the active microbial population in the processes of soil transformation under different land use in the agricultural frontier expansion may be better understood (CENCIANI et al., 2011).

It is important to highlight that Amazonian ecosystems are sensitive to land use changes (FUJISAKA et al., 1998; MARENGO, 2006; CENCIANI et al., 2011).

The native vegetation is important to the ecosystem resilience and inadequate management may cause long lasting disturbances, such as pasture degradation and desertification. Furthermore, this region is known as one of the major centers of biodiversity in the world (MARENGO, 2006) and the present study provides an impact evaluation of different land use types on the understudied microbial diversity in these soils.

At the same time this region is an important agricultural frontier, needed to supply increasing global demand for agricultural products and also provide economic growth and increased standards of live to the Brazilian population.

The aim of this thesis is to understand how microbiological processes related to the C and N cycles respond to land use change in the Southwestern Amazonia.

2.1 **Hypothesis**: Land use change from natural environment to agriculture and cattle raising results in physical, chemical, and biological soil changes that cause alterations in C, N and GGE. Soil microbial groups abundance, accessed by qPCR, may be a good indicator of these changes and of GGE.

2.2 **General objective**: To analyze microbial functional abundance by examining C, N and GHG-related genes in soils under different land use type in Southwestern Amazonia, and establishing a relationship with these biogeochemical processes.

2.2.1 Specific objectives:

a) Characterize how the soil microbial communities shift with land use change from Cerrado and Amazonian Forest to agriculture and pasture by correlating soil chemical attributes with T-RFLP (16S rRNA) and qPCR.

b) Case study to evaluate land use change, from Cerrado vegetation to agriculture and pasture, impact on C, N stocks and microbial attributes, biomass C and qPCR of the 16S rRNA gene in *Bacteria* and *Archaea* and the 18S rRNA gene in *Fungi*.

c) To test the plant effect of soybean and *Brachiaria* in mesocosms, evaluating the production of GHG (CO_2, CH_4, N_2O) and correlating with soil chemical attributes and qPCR of processes related genes (*nor*B, *nos*Z, *mcr*A and *pmo*A).

d) Evaluation of the production of GHG (CO_2, CH_4, N_2O), correlating with soil chemical attributes and qPCR of processes related genes (*amo*A, *nir*K, *nir*S, *nor*B, *nos*Z, *mcr*A and *pmo*A) in soil samples surveyed *in situ* from Amazonian Forest and conversion to pasture and agriculture.

e) To find and discuss correlations among genes abundance and soil chemical parameters related to the C, N and GHG cycles.

2.3 Thesis organization

Thesis was organized in chapters, according with the specific objectives (Figure 2.1).

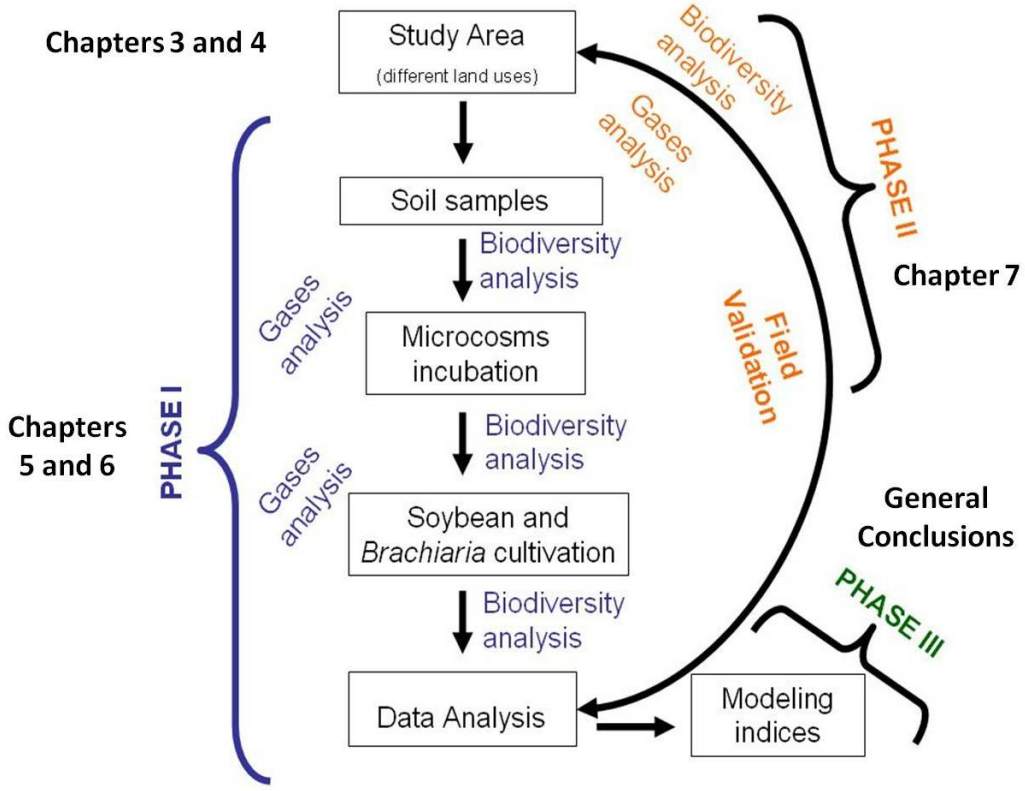

Figure 2.1 – Thesis workflow

2.4 Overview

2.4.1 Agriculture expansion in Southwestern Amazon

For Brazil, agriculture has a great social, economic and environmental importance. The country is the second largest soybean and the largest livestock producer in the world (BRASIL, 2011). Brazil has a total area of 8.502.728,269 km^2, of which 65% is still native vegetation, (25% Amazon Forest, 20% Cerrado, 20% other biomes) and of the full area, 15% is pasture and 5% agriculture (IBGE, 2011).

Due to the growing domestic and global demand for agricultural products, Brazil has dynamically expanded its agricultural activities in the past decades. Livestock and soybean cultivation started in southern Brazil and with a need for more land the agricultural frontier expanded further to the north. In the 1980´s the agricultural frontier arrived in Southwestern Amazon (Figure 2.2).

Figure 2.2 – Brazilian Vegetations and the Southwestern Amazon (Green: Amazon Forest, Yellow: Cerrado)

This expansion occurs over two of the more diverse biomes in the world, the Cerrado and the Amazon Forest. In general, land use changes in Brazil do not occur specifically in the tropical forests, but mainly in the Cerrado, or “Brazilian savanna”, which in general is not the focus of research (FRAZÃO et al., 2011).

Expansion is most intensive in the Central and North regions of Brazil (INPE, 2004), while the Southwest Amazonia region, in particular the states Rondônia and Mato Grosso, and already constitutes one of the main agricultural centers in the world. This expansion is motivated by the growing demand of soy, especially by the recently incorporated Asiatic markets. To satisfy the demand of 57 to 75 million tons of soy in 2010 (SANCHES et al., 2005), Brazil had to increase productivity and significantly expand its production area. Today, Mato Grosso State is the biggest producer of soybeans and has the biggest pasture area in Brazil (BRASIL, 2011).

Despite deforestation alarms, little is known worldwide about agriculture expansion in Brazil and it will be presented a fast summary. In Brazil, beef and large-scale grain production started mainly in Southern Brazil, expanding to the north. Beef production started in the 1500’s, with the Portuguese colonization, since the presence of the Brazilian “pampas” (native grassland) and climate was more favorable for European cattle. Soybean cultivation started in the 1970’s in the South Region, due to better adaptation of the available soybean strains to the edapho-climatic characteristics and the cultural characteristics of the people. A massive migration from Europe to Southern Brazil happened in the 1900’s, and they were more open to new crops and new technology implementation. However, with the increase of land price in Southern Brazil, coupled with an intensive Brazilian agriculture research program in the 1980’s, the soybean expanded from the South Region in direction to the Cerrado, in Central-Brazil and Amazon Forest, in Northern Brazil (BARONA et al., 2010; THE ECONOMIST, 2010).

While large-scale agriculture is focused on the international commodities, and needs high investment in infrastructure, machines, fertilizers, and pesticides, other farmers opted to invest their money in buying land for crops with lower level of inputs, like rustic pastures of *Brachiaria* spp for supporting Indian cattle. This caused the formation of two large-scale land uses, agriculture, mainly soybeans with succession of other cultures, and pasture, mainly extensive areas of *Brachiaria* spp.

In grain production, Brazil made innovations in the 1980’s with the introduction of no-till management more appropriate to tropical soils, erosion protection and

carbon sequestration in soil, and causing several soil fertility and microbiological improvements (BAYER et al., 1999). The climate conditions in Southwestern Amazon allowed other innovations, such as cultivation of two crops in the same season, usually soybean-corn. These specific managements and climate conditions make the Brazilian agriculture internationally competitive and unique (BARONA et al., 2010, THE ECONOMIST, 2010).

However, pasture cultivation presented the biggest challenge to Brazil. The low price of land, especially in the Amazon region, made it easy for farmers to buy large tracts of land, making possible economic viability by scale, but contributing to inadequate agronomical management. Brachiaria pasture corresponds to approximately 10% of the Brazilian territory, 80 Million ha (Mha), which around 30%, 25 Mha, is considered degraded (OLIVEIRA et al., 2004).

2.4.2 Carbon, Nitrogen and GHG cycles in soils

Several works review the importance of the carbon and nitrogen cycles for soils, both are complex cycles and present tremendous importance for life (BAYER et al., 1999; MOREIRA; SIQUEIRA, 2006; CANFIELD et al., 2010). They are related to the maintenance of energy flow and matter recycling in natural environments and of fundamental importance in optimizing agricultural soil management.

While the cycles balance can result in biomass gain for the natural or agricultural systems, they can also produce GHG and pollutants such as nitrate (Figure 2.3). The chemical balance of the key points of the cycles can bring natural ecosystems benefit or can be of benefit to agricultural systems (BAYER et al., 1999; MOREIRA; SIQUEIRA, 2006; CANFIELD et al., 2010).

In relation to the carbon cycle, benefits include carbon accumulation in soil, which can increase humic substances and consequently has positive effects for soil fertility, such as Cation Exchange Capacity (CEC), nutrient cycling, soil structure, erosion protection and water retention (BAYER et al., 1999; MOREIRA; SIQUEIRA, 2006; CARVALHO, 2010).

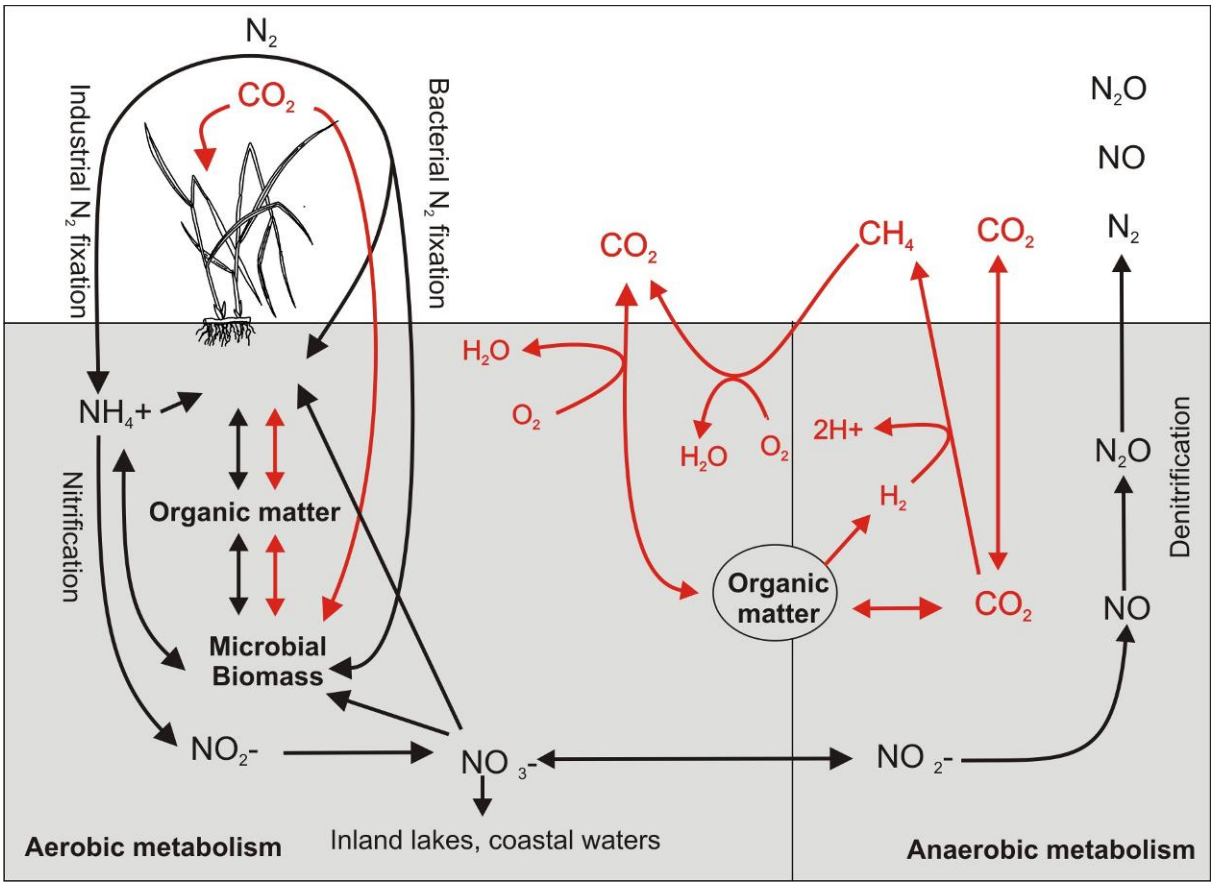

Figure 2.3 – Summary of the carbon and nitrogen cycles in the soil

In relation to the nitrogen cycle, benefits can be related to nitrogen fixation and mineralization of nitrogen to be used by plants and consequently in the food web (MOREIRA; SIQUEIRA, 2006). In agriculture, several benefits can be achieved. Nitrogen is one of the most important nutrients, usually added as fertilizer, so optimization of key points, such as the biological fixation can reduce economic costs and also the environmental impacts of production and transportation of N-fertilizers. A better understanding of the nitrification and denitrification cycles can result in optimization of N-fertilizer use, reducing costs and reducing of GGE and nitrate leaching to the environment (MOREIRA; SIQUEIRA, 2006).

Excluding fire, where gas emission is an essential chemical and physical process, in all agricultural and cattle activities GGE are related to living organisms. In the trophic chain, primary producers produce organic matter mainly by CO_2 fixation through the photosynthesis process and this matter is reused as a source of carbon and energy by other organisms (MOREIRA; SIQUEIRA, 2006).

Under aerobic conditions the production of CO_2 results in a neutral balance between fixation (photosynthesis) and gas release (aerobic respiration). In some cases, this balance may be positive (more fixation than CO_2 production), and is called CO_2 sequestration. However, native vegetation and soil are the largest carbon reservoir in the form of organic matter, which is accumulated over a long period of time. In land use change, this accumulated carbon may be released to the atmosphere as CO_2 in aerobic conditions, especially by organic matter oxidation by microorganisms.

Under anaerobic conditions, organic matter oxidation may produce CH_4 and N_2O gases that global warming potential of 23 and 296 times higher than CO_2, respectively (IPCC, 2007). In Brazil, different studies quantified and estimated the GGE in agricultural systems; however, none of these studies studied the diversity of organisms that produce these gases (NEILL et al., 1997; CARVALHO, 2010).

Land use change impact both cycles. When conservationist practices are not adopted, intensive land use invariably leads to negative effects on both environment and agricultural productivity (CERRI et al., 2004; FOLEY et al., 2005). The reduction of soil organic matter is followed by gas emissions (especially by CO_2, CH_4 and N_2O) to the atmosphere, enhancing global warming. Additionally, it can be related also with modification in the quality of the remaining organic matter. These modifications occur, for example, as soil disaggregation leads to erosion losses, and reduction of nutrient availability to plants and lower water storage capacity. These and other factors negatively affect agricultural productivity, with reduced food productivity, and reduced sustainability in the soil-plant-atmosphere system (LAL, 2003; SIX et al., 2004; KNORR et al., 2005). So, the uses of adequate management techniques, and a better knowledge of these processes, are essential to environmental sustainability, agriculture efficiency, and costs reduction.

2.4.3 Gene fingerprinting and abundance as indicator of the cycles

Our current lack of comprehensive knowledge of microbial diversity in environmental samples is due to the use of traditional methods for the isolation and cultivation of microorganisms under laboratory conditions. Only a small fraction of bacteria, approximately 0.1% of total population, can be cultivated using traditional microbiological techniques (VAN ELSAS et al., 2010).

Recent advanced studies in the field of molecular biology (DNA extraction, PCR amplification, DNA cloning and sequencing of marker genes) permitted the development of techniques to identify microorganisms without isolation and cultivation. Total soil DNA may be extracted and analyzed by a variety of molecular techniques, such as combination of PCR and qPCR analyses of a gene of interest and sequencing, or the creation of fingerprint patterns by techniques such restriction fragment length polymorphism (T-RFLP). It is evident the better resolution of high-throughput sequencing techniques, but due to high costs, fingerprinting techniques are still wide used (VAN ELSAS et al., 2011).

The analysis of T-RFLP determines the terminal restriction fragment length polymorphism of an amplified PCR product, since one primer is labeled with a fluorescent marker. This is a very sensitive method to study soil bacterial structure; furthermore, it has the capacity of analyzing a large number of samples. This analysis results in DNA-fingerprinting patterns that can be quantified and interpreted according to the soil microbial community structure in other samples or in T-RFLP databases (LIU et al., 1997).

Quantitative PCR was recently developed with the objective to estimate the number of copies of a gene of interest (HEID et al., 1996; DÖLKEN et al., 1998). The amount of fluorescent signal is directly correlated to the quantity of the produced product in real time. Comparison to standards during amplification allows quantification of gene copy numbers in an environmental sample (HEID et al., 1996; DÖLKEN et al., 1998).

While measurements of C and N microbial biomass are some of the most widely used techniques in soils, it cannot separate the functional groups within a sample. Using qPCR it is possible to separate groups of interest within a sample (Figure 2.4).

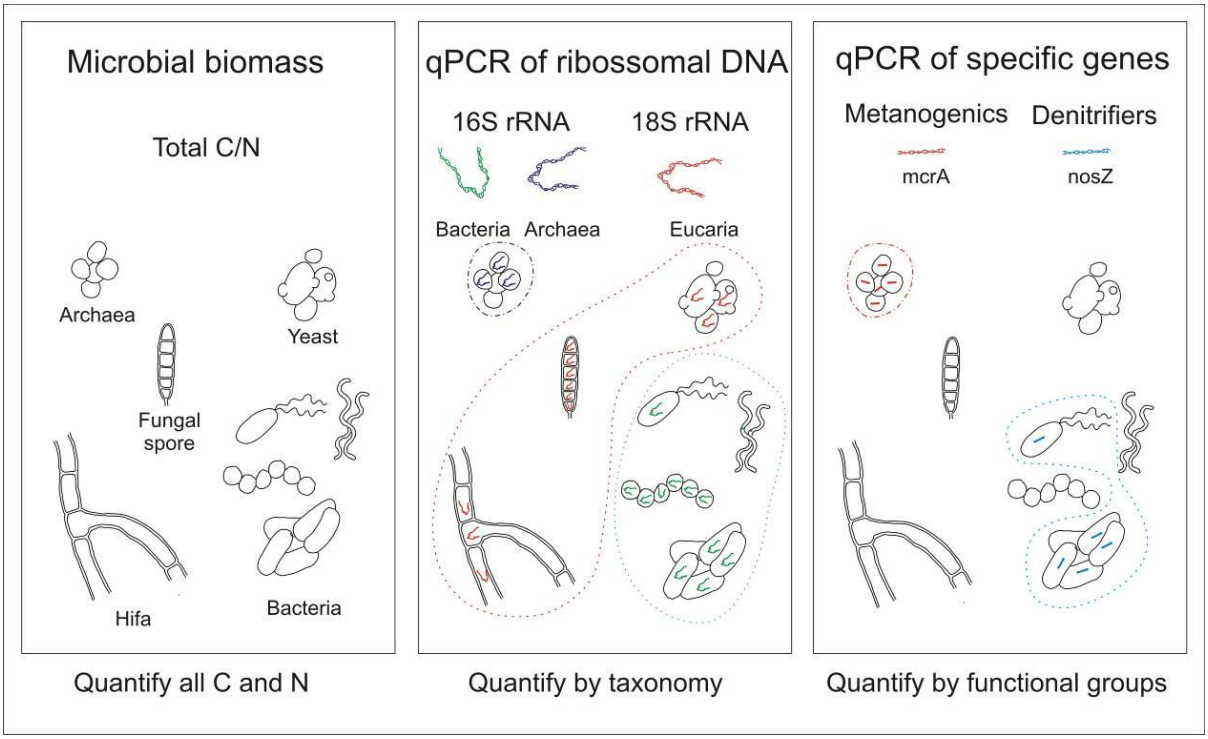

Figure 2.4 – Comparison of microbial biomass versus the qPCR technique

Quantitative PCR permits quantification of active microbial populations in the soil by measuring processes related genes. As example, his technique was used to study ammonium oxidation and denitrification, indicated by the gene *amo*A present in ammonium oxidizers (INNEREBNER; KNAPP, 2006, LEININGER; URICH, 2006), and the genes nirK and nosZ, present in denitrifying bacteria (HENRY et al., 2006), respectively.

2.5 Genes related to the C, N and GHG cycles

Since all biogeochemical processes are catalyzed by enzymes, it is possible to select genes related to these enzymes to use as indicators of the processes (CANFIELD et al., 2010).

Gene choice is based on the importance of the gene in the constitution of the enzyme and the abundance of gene sequences available to allow for good primer design. Based on this information, we can plan an experimental model to quantify chemical components and microbiological components, represented as genes (Figure 2.5).

The 16S rRNA gene transcribes the smaller ribosome subunit in prokaryotes. This gene has conserved regions and variable regions, and nucleotide base substitutions are proportional to the evolution of prokaryotic species, making this gene an “evolutionary clock”. This gene has been widely used in studies of diversity and taxonomy of prokaryotes (COHAN, 2002). Primers can be used to differentiate between Bacteria and Archaea in environmental samples and 18S rRNA gene primers can be used to detect fungi (VAN ELSAS et al., 2011). Several genes are involved in the carbon cycle and it is impossible to study all by qPCR. For CO_2 production, we think that the ribosomal genes can give an idea of total microbe abundance, which may be related to microbial activity.

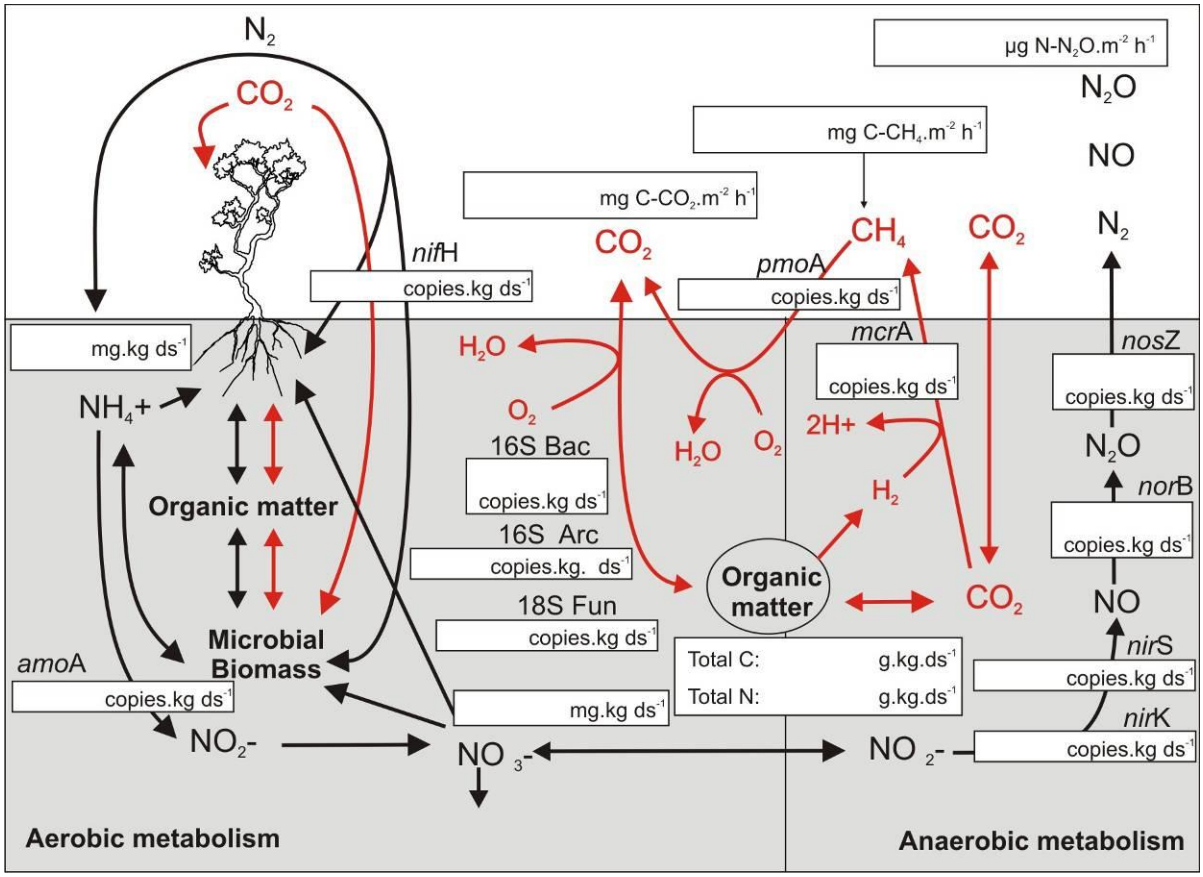

Figure 2.5 – Suggested model to study the chemical and microbiological aspects of the C, N and GHG cycles

The gene *mcr*A encodes the protein McrA, the alpha subunit of methyl coenzyme M reductase, responsible for the reduction of methyl-coenzyme M (2-(methylthio) ethanesulfonic acid) and 7-mercaptoheptanoylthreonine phosphate to methane and heterodisulfide and catalyses the oxidation of a range of substrates

including ammonia, methane, halogenated hydrocarbons and aromatic molecules. This gene is sufficiently conserved and consistent with 16S rRNA phylogenies to allow the identification of methanogenic microorganisms in environmental samples (DHILLON et al., 2005).

The gene *pmo*A encodes the methane monooxygenase A subunit. It catalyses the oxidation of a range of substrates including ammonia, methane, halogenated hydrocarbons and aromatic molecules. It is responsible for the methane oxidiation by methanotrophic bacteria (SVENNING et al., 2003). The study of this gene is of interest for investigating interactions between methanogens and methanotrophic bacteria and the related balance between methane production and consumption.

The gene *nif*H encodes the protein NifH, nitrogenase reductase (an Fe-protein). The key enzymatic reactions in nitrogen fixation are catalyzed by the nitrogenase complex, which has 2 components: the iron protein and the molybdenum-iron protein. Several studies have targeted this gene (CANFIELD et al., 2010).

The gene *amo*A, encodes the protein AMO, ammonia monooxygenase. It oxidizes ammonia to hydroxylamine, an intermediate in the ammonia oxidation pathway and is responsible for the first stage of transformation of ammonium to nitrate. Several studies have examined the function of this gene in soil (LEININGER; URICH, 2006).

The gene *nir*K, encodes the protein NirK, a copper-containing nitrite reductase (NO-forming), which catalyzes the conversion of nitric oxide to nitrite.The gene *nir*S, encodes the protein NirS, a cytochrome cd1 nitrite reductase precursor/ hydroxylamine reductase, responsible for the reduction of respiratory nitrite to NO. Both *nir*K and *nir*S are used to target denitrifiers in soil (HENRY et al., 2006).

The gene *nor*B, encodes the protein NorB, nitric-oxide reductase subunit B. It is a component of the anaerobic respiratory chain that transforms nitrate to dinitrogen (denitrification). NorB is the catalytic subunit of the enzyme complex and shows proton pump activity across the membrane in denitrifying bacterial cells. The mono nitrogen reduction is probably coupled to electron transport phosphorylation. Some works focus on the function of this gene in soil (DANDIE et al., 2007).

The gene *nos*Z, encodes the protein NosZ, nitrous-oxide reductase, which is part of a bacterial respiratory system activated under anaerobic conditions in the presence of nitrate or nitrous oxide. It is a key gene, since it is responsible for the

reduction of the potent GHG nitrous oxide to dinitrogen. Several studies have focused on this gene (HENRY et al., 2006; DANDIE et al., 2007).

All these genes may be used as indicators of these processes, and their quantification by qPCR can be related to abundance of the microbial groups in soil (DANDIE et al., 2006; MORALES et al., 2010). Most of the genes have constitutive expression, what means that on average, since different organisms could have different protein concentrations, gene abundance can be related with protein synthesis (Table 2.1).

Most of these genes are related to anaerobic processes, so detection of the gene means presence of the organism and the expressed protein in the environment. Aerobic conditions in the soil can change fast, as a strong rain after a dry period. It means that most of the anaerobic respiration key proteins must be ready to use, even in a basal level, since it means survival and it is a strong selection factor. Most of the protein and expression levels were carried out in microbial isolates in laboratory conditions. Little is known about expression of these genes and activity of the proteins in the environment.

Also, little is known about taxonomy distribution in the environment, with most of the sequences and distribution in the different microbial domains related to knowledge based on isolates. Based on a GenBank search (19th October, 2011), the presence of the genes in each domain was estimated. Most of the sequences available are related to bacteria or archaea, emphasizing the importance of both groups to the C, N and GHG related processes (Table 2.1).

In relation of copy number per cell, little is still know. So it is difficult to establish straight relations between copy number and cell abundance. For example, for the gene 16S rRNA, it can vary from 1 to 21 copies, but 40% of the known species present one or two copies and only 10% more than 7 copies per cell (KANG et al., 2010). For the other genes information is still scarce, and need to be analyzed genome by genome (Table 2.2).

Table 2.1 – Description of genes with potential to be used as indicators of the biogeochemical processes

Gene[1]	Expression	Predicted Functional Partners	Location in the Cell	Bacteria (%)[2]	Archaea (%)[2]	Fungi (%)[2]
nifH	σ54 -dependent	NifD, NifK, NifB, NifE, NifN, NifX, FixU, NifQ	Cytoplasm	98.5	0.6	0.9
amoA	Constitutive (σ70)	Hao, AmoB, AmoC	Transmembrane	41.2	58.8	0.02
nirK	Aerobic, NO_2 and pH dependent	NorB, NorC, NorD, NorE, NorQ, NapA, NasA	Periplasm, transmembrane	97.7	2.2	0.1
nirS	Constitutive and regulated by NO and O_2	nirC, NorB, NorC, NirJ, NirF, NapA, NirH, NirG, NirL, NirD	Periplasm, transmembrane	98.4	1.4	0.2
norB	Constitutive and regulated by NO and O_2	NorC, NorD, NorQ, NorZ, NirS, NirO, NirF, NirN, NirC, NnrS	Transmembrane	97.8	0.8	1.4
nosZ	Constitutive	NosD, NosY, NosR, NosF	Transmembrane	99.8	0.2	0
mcrA	SOS-inducible cryptic prophage element e14	McrB, Mrr, YmfD, YagL, YhcA, GltF, IraM, YfjK, YigA, YhcF.	Cytoplasm	9.4 [3]	90.4	0.1 [3]
pmoA	constitutive expression and regulated by acetate/methane	MxaI, MxaF,	Transmembrane	95.6	4.4	0

[1] Table based on NCBI, Expasy, Pfam, String and Smart genes/proteins databases.
[2]The matches that do not correspond to the expected product were excluded. The unclassified genes were also excluded from the analysis. Possible annotation or taxonomic classification errors may be present, as the methodology was not able to discard these errors. [3] *mcr*A abundance could be an annotation error in GenBank, since it is expected only in Archaea.

Discussing an average number of copies of genes per cell is still speculative, since a full characterization of the microbe diversity in soils is still not possible. For such characterization, it would be needed to sequence the full genome of all bacteria present in the samples, which means using of high throughput sequencing, which is still expensive.

To the best of our knowledge, up to now, no metagenomic sample from soil was completely sequenced, so, it is not possible yet to infer influence of gene copy numbers in the comparisons. In this thesis, discussion will focus as copy number per cell is in average equal among the studied areas.

Table 2.2 – Number of copies of genes per organism

Gene	Copy numbers per cell	Reference
16S rRNA Bacteria	1-21	KANG et al. (2010)
16S rRNA Archaea	Probably like Bacteria	-
18S rRNA Fungi	Several, various nucleus per organism	-
amoA	1-3	HALLIN et al. (2009)
nirK	1-3	HALLIN et al. (2009)
nirS	1-3	HALLIN et al. (2009)
norB	1-2	JONES et al. (2008)
nosZ	1-3	HALLIN et al. (2009)
mcrA	1-2	FREITAG; PROSSER (2009)
pmoA	1-2	FREITAG; PROSSER (2009)

All this genetic, proteomic and physiological information is of tremendous relevance in explaining microbial activity in the environment and biogeochemical processes. A better knowledge of these microbial processes will allow us to create strategies to optimize land use management and reduce environmental loses in these biogeochemical processes (RICHARDOSON et al., 2010).

2.5.1 Study Sites Description

Our study was carried out in the Brazilian State of Mato Grosso. Two municipalities were chosen, one in the Cerrado region, Campo Verde, and one in the Forest region, Sinop (Figure 2.6). Both regions stand out as important agricultural sites in Southwestern Amazon.

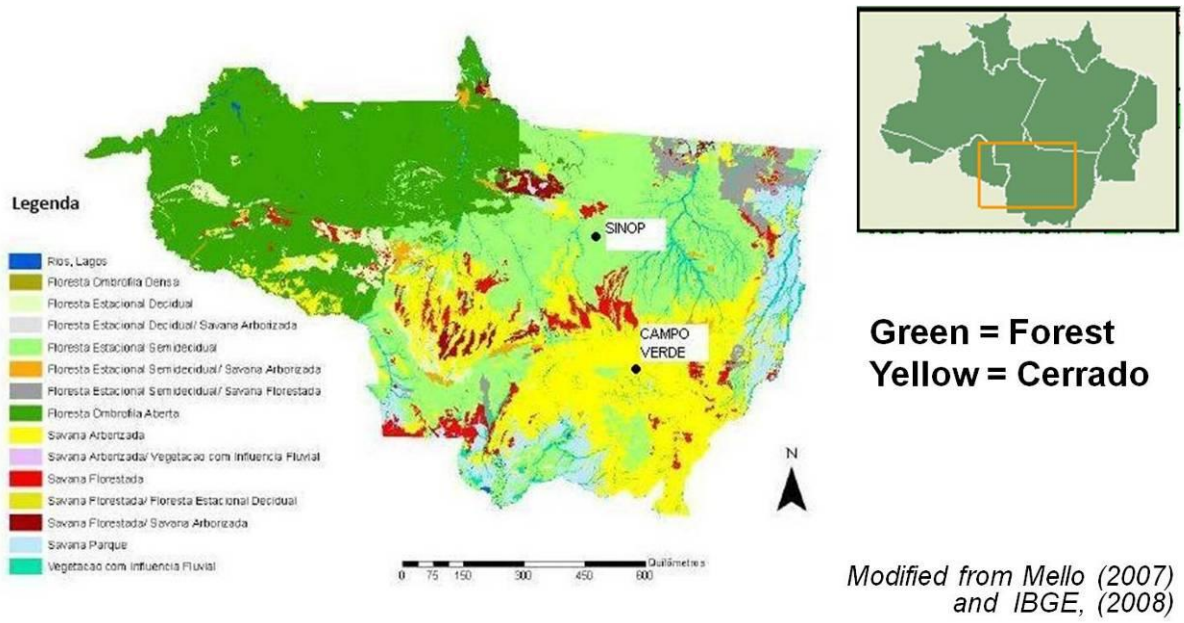

Figure 2.6. Vegetation map of Mato Grosso State and the two municipalities chosen for this study

The Cerrado is the second largest biome in Brazil, occuping an area of 2,036,448 Km^2 and accounting for over 20% of the territory. The Cerrado has a hot sub-humid tropical climate with a dry season and a rainy season and a mean annual temperature between 22°C and 27°C. The landscape of the Cerrado is predominantly characterized by extensive savanna formations intercepted by woods along rivers, in valley bottoms. In the Cerrado more than 1,500 animal species have been identified, forming the second largest diversity of animals in the world (IBF, 2011).

The Amazon rainforest covers an area of 4,196,943 Km^2, representing almost 50% of Brazilian territory. It has a hot and humid climate (average temperature of 25°C). The Amazon is made up of distinct ecosystems with dense forests, seasonal

forests, *igapó* forests, floodplains, flooded fields, savannahs, mountain refuges and pioneer formations. It is estimated that this biome harbors more than half of all living species in Brazil. Even being the most preserved Brazilian biome, about 16% of its area has been devastated (IBF, 2011).

2.5.2 Experimental design and farm choice

For the experimental design, we chose a randomized study, composed of three replicate sites for each land use in each vegetation type (Figure 2.7). The replicate sites were not considered as blocks, since replicate sites were chosen randomly, without edapho-climatic reasons to separate them in blocks. So we used a full randomized design. Inside each replicate site, samples were taken from three to ten soil cores (pseudo replicates), depending on the study and objective. The number of pseudo replicates and objectives will be detailed in each chapter.

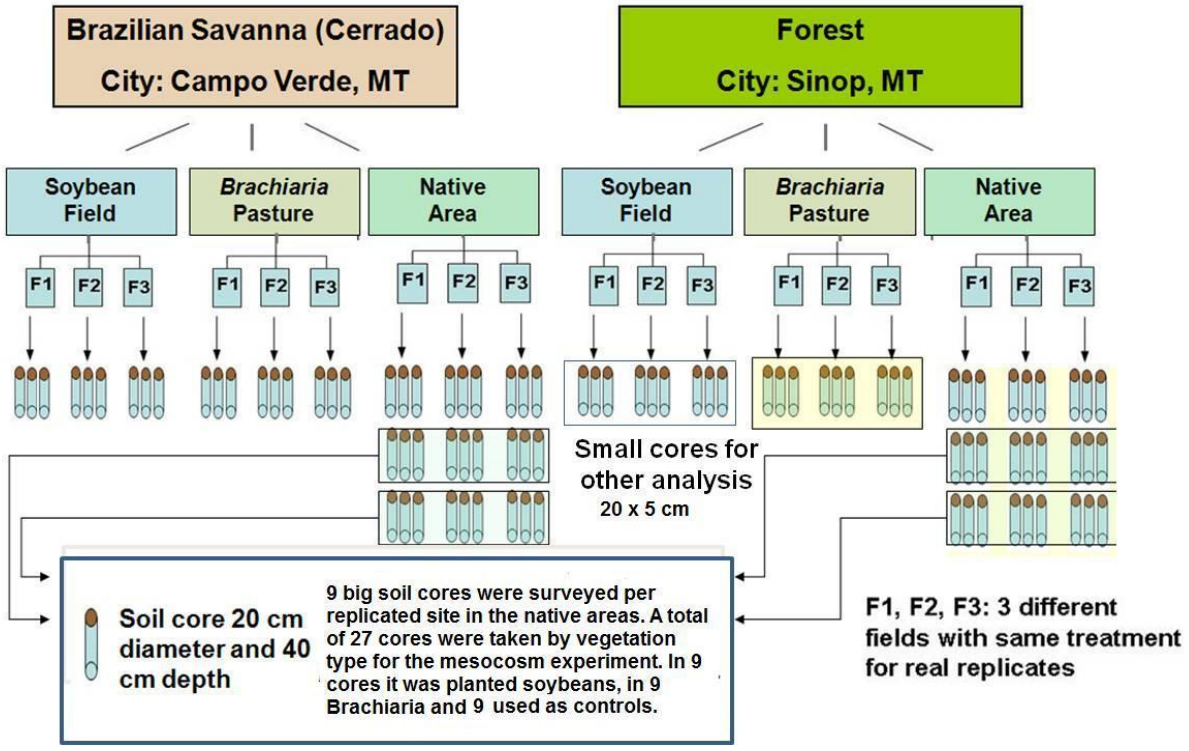

Figure 2.7 – General survey design. Number of soil cores surveyed varied from five to ten cores, depending on the study

A short description of each chapter study is described as follow. For the first study, five soil cores were characterized to compare Cerrado and forest vegetations to agriculture and pasture (Chapter 2). For the second study, ten soil cores in four depths per land use were characterized from Cerrado areas, to examine carbon and nitrogen stocks, soil chemical and microbiological parameters (Chapter 3). Nine soil cores per replicate site were taken to construct the first mesocosms study (Chapter 4) and three for the second (Chapter 5). To compare functional genes in the field, we concentrated on one municipality, Sinop. Chambers for GHG measurement were installed in five distinct points per replicate site (five replicates), with one soil core per chamber. Two replicate sites were tested per land use, on three different days to reduce temporal variability (Chapter 6).

To randomize the sampling, a grid of 100 m x 100 m was made and subdivided in 10 x 10 m plots (Figure 2.8). Each grid was composed of 10 rows and 10 columns, resulting in a total of 100 plots. Using a 10 sides dice, 10 plots were random chose inside each plot. In the first dice roll, one of the 10 rows was selected, in the second roll, one of the 10 columns was selected. The 10 plots were enumerate from 1 to 10 and used in the different studies. We chose to use the same random grid to all the different replicate sites, so comparisons would be made among the different chapter, and spatial assumptions, if relevant, discussed.

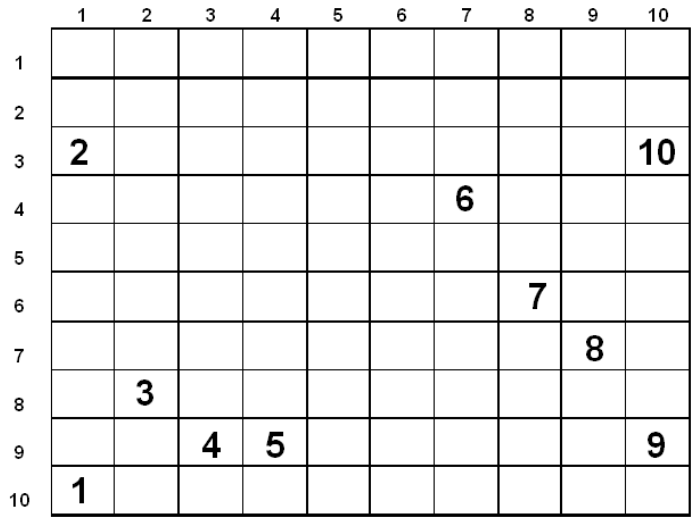

Figure 2.8 – Grid used in the different replicate sites to soil survey. Each number corresponds to a random plot selected by dice

Two main farms were chosen, Marabá Farm, owned by Mr. José Puppin, in Campo Verde and Luiza Farm, owned by Mr. Adelmo Zuanazzi in Sinop. Both properties had native areas cleaned in the 1980s (the start of soybean planting in southwestern Amazonia) with owners willing to participate in studies and available information on the history of the areas. However, since the soil of Cerrado area in Marabá Farm had a different, sandier, texture than that of soy and pasture soils, we decided to collect the soil of a *cerrado* in an adjoining property in order to make the most appropriate comparison between the areas. The name of the adjoining farm is São João Batista, owned by Mr. Jader Bergamasco. The Luiza Farm, in Sinop, had no pasture area. However there was an adjacent property, exclusively used for livestock that was chosen for sampling, the farm of Mr. José Alves da Silva.

An additional farm, Jaguaruna farm, was chosen near Sinop, in the municipality of Porto dos Gauchos, where new areas were being cleared. Soil from this farm was used for one of the mesocosms experiment (Chapter 4) and also for the comparison of genes and GHG in the field (Chapter 6).

2.5.3 Land use history and characteristics of the study sites

In the agricultural site we focused the surveys on soybean management and pasture, the two biggest uses of agricultural land in Southwestern Amazon. In the past, soybean was cultivated in monoculture in this region, but since the 2000's with adaptation of no-till systems and looking for a higher economical return, most of the farmers adopted crop rotation with succession cultivation after the soybean.

In the farms that adopt succession cultivation, the sowing of the next crop occurs immediately after soybean harvest, usually at end of January or beginning of February. Sowing depends on growth of the soybean seedling, which depends on the start of the rainy period, usually between the middle of September to the end of October (Figure 2.9).

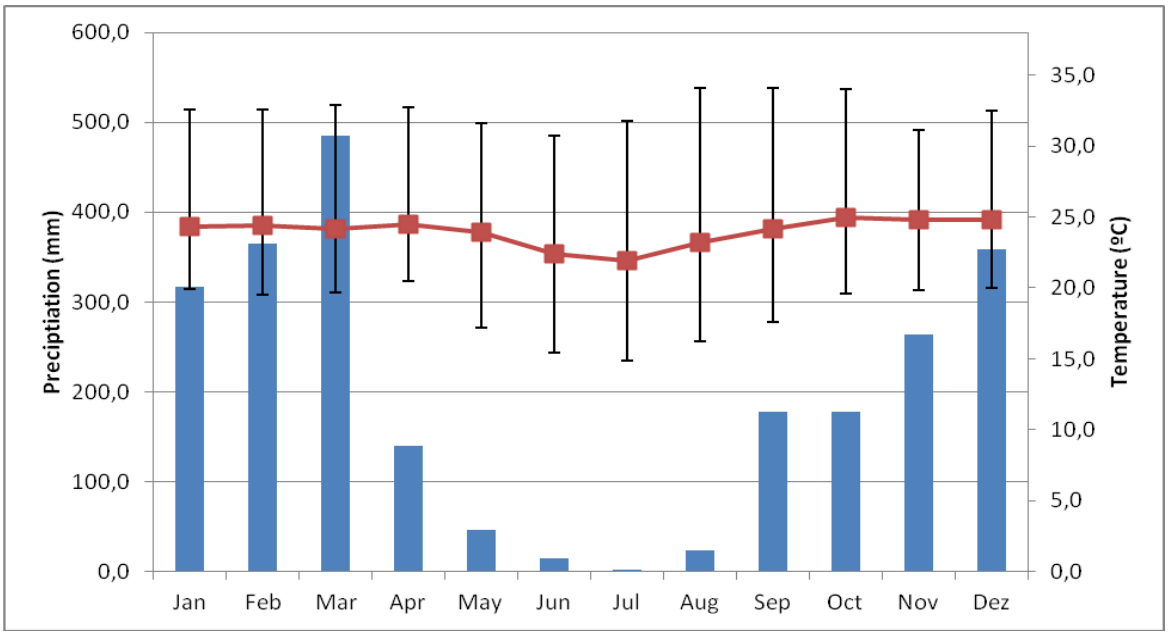

Figure 2.9 – Average precipitation and average temperature (bars represents maximum and minimum) in Vera, MT (EMBRAPA, 2003). This city is located between Sinop and Campo Verde. No available information for the study areas

Crop choice depends on the rain history of the farm region and on economic considerations (Figure 2.9). Most common succession crops are corn, sorghum, millet and the pasture grass species *Brachiaria* (Agriculture-Live Stock integration systems). In the study sites, the farms have cultivated different crop in different years (Table 2.3).

In this thesis, it was not analyze the specific effect of succession in the study sites, but it is expected that it contributed some interference to the microbial community present during soybean cultivation.

Forwarding this study, we treat the agricultural fields as soybean, since it was the predominant crop in the area and we surveyed the soils during soybean growth. The effect of the succession cultivation must be kept in mind and should be studied in future works.

Table 2.3 – Land use history and sites characteristics

Characteristics	Amazonian Forest sites			Cerrado sites	
Vegetation	Ombrophilous Forest			Cerrado *sensu stricto*	
Climate	Aw (Köppen-Geiger)			Aw (Köppen-Geiger)	
Annual Precipitation	~2000 mm			~2000 mm	
Annual Temperature	~25.0 ºC			~23.0 ºC	
Soil type	Red Oxisol			Red Oxisol	
Soil texture	Clay			Clay	
Municipality	------- Sinop -------		Porto dos Gauchos	Campo Verde	
Land use	Agriculture	Pasture	Agriculture	Agriculture	Pasture
Farm name	Luiza	Sao Joao	Jaguaruna	Maraba	Maraba
Name in the Thesis	Soybean 25y	Pasture	Soybean 2y	Soybean	Pasture
Year of Deforestation	1978	1988	2008	1979	1975
Old land uses	Guarana, Coffee	*B. humidicula*	Forest, Rice	Cerrado	Cerrado
Soybean or pasture since	1986	1988	2009	1979	1975
Recent use history:					
Succession 2000/2001	Soybean	*B. brizantha*	Forest	Soybean	*B. brizantha*
Succession 2001/2002	Soybean	*B. brizantha*	Forest	Soybean	*B. brizantha*
Succession 2002/2003	Soybean	*B. brizantha*	Forest	Soy/Corn	*B. brizantha*
Succession 2003/2004	Soy/Sorghum	*B. brizantha*	Forest	Soy/Corn	*B. brizantha*
Succession 2004/2005	Soy/Corn	*B. brizantha*	Forest	Cotton	*B. brizantha*
Succession 2005/2006	Soy/Sorghum	*B. brizantha*	Forest	Soy/Corn	*B. brizantha*
Succession 2006/2007	Soy/Sorghum	*B. brizantha*	Forest	Soy/Corn	*B. brizantha*
Succession 2007/2008	Soy/Millet	*B. brizantha*	Forest	Cotton	*B. brizantha*
Succession 2008/2009	Soy/Corn	*B. brizantha*	Rice	Soybean	*B. brizantha*
Succession 2009/2010	Soy/Corn	*B. brizantha*	Soybean	NA	*B. brizantha*
Succession 2010/2011	Soy/Corn	*B. brizantha*	Soybean	NA	*B. brizantha*

2.5.4 Marabá Farm, Campo Verde, Cerrado

In Campo Verde, the farm has about 20000 hectares (ha) (~60000 acres), 9000 ha (27000 acres) with cotton, 7000 ha (21000 acres) with soy and 1000 ha (1200 acres) with pasture. Agricultural activities started in 1975 with the removal of native vegetation and establishment of pasture. From 1981 to 2000soy was planted as a monoculture, then a rotation of soy with cotton and maize was introduced, in the management style typical of the region, to optimize land use and increase profitability to the farmer. The older plots on the property were chosen for sampling. The centrals geographic coordinates of the areas are S 151588.8 550700.0 W (soy), S 151601.9 550748.8 W (pasture) and S 152228.7550542.8 W (Cerrado area in neighboring property) (Figures 2.10 and 2.11).

Figure 2.10 – Top: Area of pasture, soybeans and cerrado sampled in Campo Verde. Middle: Cerrado next to a newly planted cotton crop. Bottom: A pickup truck is ahead of the cerrado giving an idea of the height of vegetation (January, 2009)

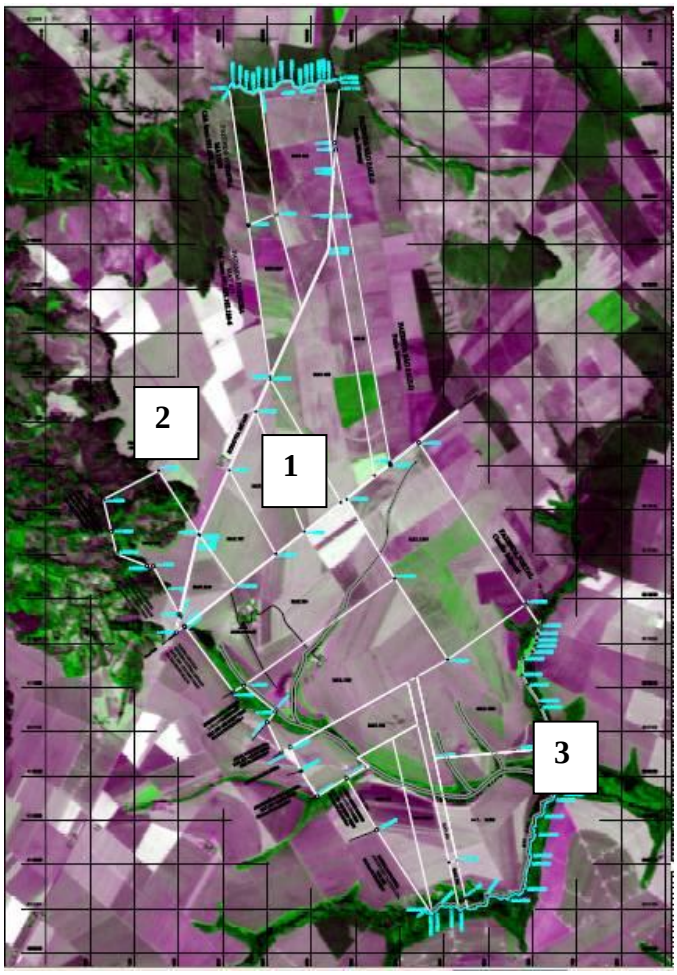

Figure 2.11 - Satellite photo of Maraba Farm. Soy (1), pasture (2) and neighboring property (3)

2.5.5 Luiza and Sao Joao Farm, Sinop, Forest

Luiza Farm presents about 1600 ha (650 acres) of soy, planted since 1986. Soy was planted in the monoculture system until 2004 when soy was planted in succession with corn, sorghum or millet, in the typical management of the region. The pasture area (neighboring farm) was planted from 1988 with *Brachiaria humidicola*, and *Brachiaria brizanha* from 1996. Older, adjacent parcels between the different land uses were chosen for sampling and the soil under forest was collected from the two farms. The central geographic coordinates are: S 120521.1 552824.1 W (soy), S 120553.3 552846.0 W (forest) and S 120535.6 552838.2 W (pasture area in the neighboring property) (Figures 2.12 and 2.13).

Figure 2.12 - Area of pasture (top), soybeans (middle) and forest (bottom) in Sinop. A pickup truck is ahead of the forest, giving an idea of the height of vegetation (January, 2009)

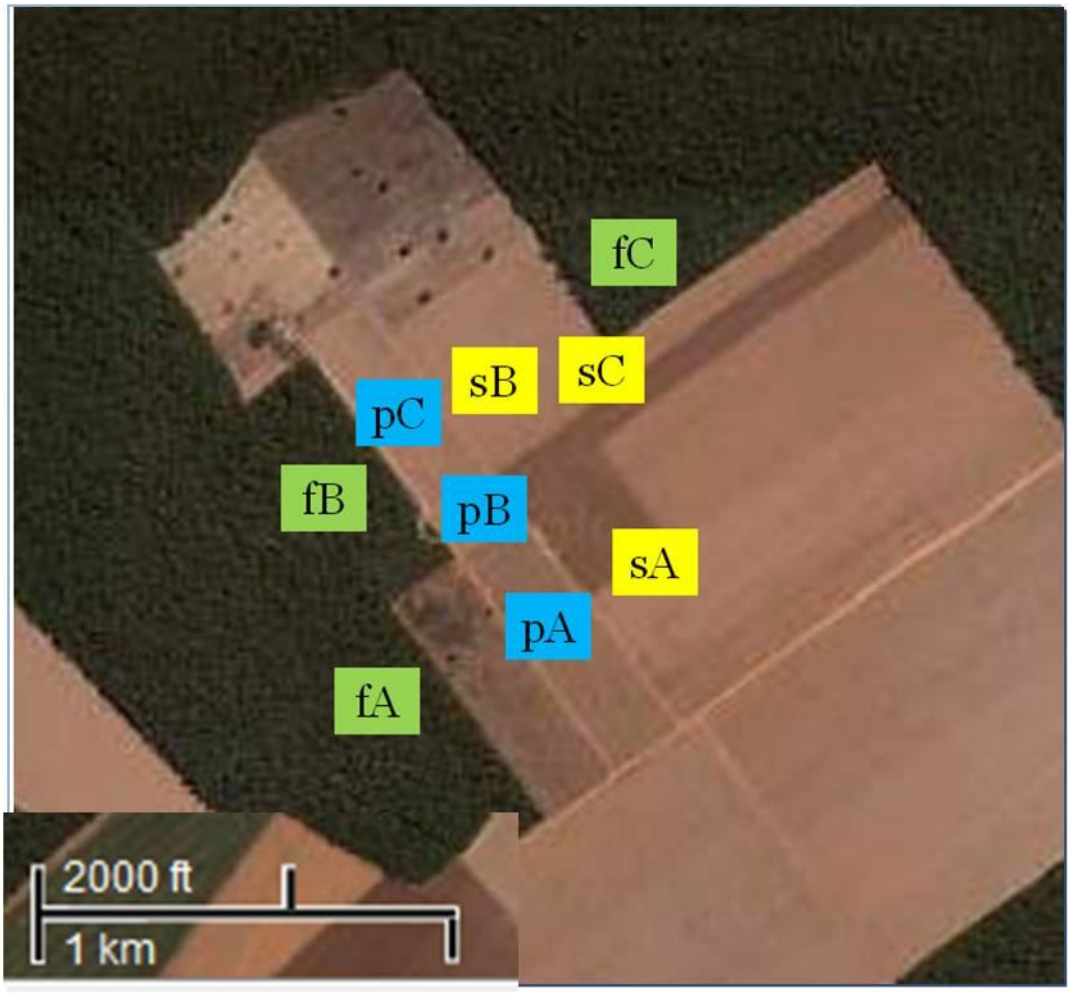

Figure 2.13 - Satellite photo of the areas in Sinop, forest (fA, fB, fC), pasture (pA, pB, pC) and soy (sA, sB and sC)

2.5.6 Jaguaruna Farm, Forest

This farm is located in Porto dos Gauchos, near the municipality of Sinop. The study area corresponds to an area of 1000 ha (~ 3000 acres), and it was deforested in 2008 when it was cultivated with rice. Vegetation was mechanical removed and the area subsequently cleared by burning. For further soil preparation, a disc harrow was used twice to incorporate remaining roots and dolomite lime, which was applied at an average rate of 5.6 Mg per ha. Rice was cultivated, with application of 400 $kg.ha^{-1}$ of the fertilizer 04-14-08 ($N\text{-}P_2O_5\text{-}K_2O$) and 50 kg of urea. Usually rice is planted for the first years of cultivation, since it is tolerant to lower pH. Since November 2009 soybean has been cultivated (Figure 2.14).

Figure 2.14 – Soybean and native Forest at Jaguaruna Farm, Porto dos Gauchos, MT. Photo was taken the second year of cultivation, 2010/2011

The coordinates of the replicate sites are, site A, S 0581360; W 8701944 (soybean and adjacent forest), site B, S 0580376; W8701914 (soybean and adjacent forest) and site C S 0579201; W 8701860, (soybean and adjacent forest).

3. MICROBIAL COMMUNITY STRUCTURE OF FOREST AND CERRADO SOILS AFTER CONVERSION TO SOYBEAN FIELDS AND *BRACHIARIA* PASTURE

Abstract

The Southwestern Amazon region is the most dynamic agricultural expansion frontier worldwide today. Tropical forests and Cerrado are converted rapidly to pasture and agriculture. Following deforestation, soil C and N can be lost to the atmosphere in the form of greenhouse gases (CO_2, CH_4 and N_2O) and numerous chemical changes occur in the soil as a result of fertilization and agricultural management. Changes are also expected in the microbial structure under the different land uses. The objective of this chapter was to compare microbial structures, in Southwestern Amazonian Cerrado and Forest soils following land use changes to agriculture (soybean – *Glycine max* L. Merrill) and pasture (*Brachiaria brizantha* A. Rich.). Microbial structure was accessed by Terminal Restriction Fragment Length Polymorphism (T-RFLP), and the quantification of target genes by qPCR. Soil samples were taken from the three different land uses (native vegetation, soybean field, and pasture), and in two different municipalities. Sinop harbors a tropical forest and Campo Verde is characterized by Cerrado In both sites pasture and soybean fields were established more than 20 years ago. Chemical changes concurrent with land use changes were increases of soil pH, Ca, Mg, P, K, and N concentrations. Microbial structure was also affected by change in land use, with Cerrado and Forest more closely related to each other than Agriculture, and Pasture was intermediate among them. No loss in microbial richness was observed; TRFs abundance was statistically the same for all the areas. Gene abundance was different in the distinct land uses, and in general, the Cerrado areas harbored the highest abundances. Based on the molecular results, land use changes influenced significantly the structure of microbial communities in the Southwestern Amazonian soils. Further use of molecular investigation may help to detail the differences. A PCA analysis indicated the correlation among the soil chemical and biological attributes, showing that microbial structure responded to the environmental changes caused by land use change. Several trends suggest that Cerrado areas present higher microbial abundance and activity.

Key words: Land use change. T-RFLP. qPCR.

3.1 Introduction

The Southwestern Amazon is the region with the highest dynamic agriculture expansion worldwide today. Tropical forests and Cerrado are converted to pasture and agriculture. Following deforestation, soil C and N can be lost to atmosphere in the form of greenhouse gases (CO_2, CH_4 and N_2O) and several chemical changes occur in soil due to fertilization and agricultural management. Changes are also expected in the microbial structure in the soil from the different land uses.

The objective of this chapter was to compare microbial structures and abundance in Southwestern Amazonia Cerrado and Forest soils following land use changes to agriculture (soybean – *Glycine max* L. Merrill) and pasture (*Brachiaria brizantha* A. Rich.) by Terminal Restriction Fragment Length Polymorphism (T-RFLP) and qPCR.

3.2 Material and Methods

3.2.1 Survey

Soil samples were taken from the three different land uses (native vegetation, soybean field and pasture), in two cities: Sinop (Tropical Forest - S 120553.3 W 552846.0) and Campo Verde (Cerrado - S 151588.8 W 550700.0); where pasture and soybean fields were established more than 20 years ago (Chapter 2).

In each area, three replicate sites were chosen from each land use (real replicates), and five plots in each site (pseudo-replicates) were selected and individual soil samples were taken from 0-17 cm soil depth.

3.2.2 Chemical analysis

Soil samples were processed as described by Carvalho et al. (2007). First, samples were air-dried and sieved at 2 mm. Soil pH was measured in 0.01 M $CaCl_2$. Exchangeable cations (Ca^{2+}, Mg^{2+} and K^{+}) and available P were extracted by using ion exchange resins and trace elements by microwave extraction, according to Van Raij and Quaggio (1983). From each sample 10 g was ground and sieved to 0.25

mm for determination of C and N, using a Carbon Nitrogen Analyzer-LECO CN-2000 (CARVALHO et al., 2009).

Litter composition was determined by the CN method (CARVALHO et al., 2009).

Total DNA was extracted from 0.25 g of the soil samples using the Power Soil kit (MoBio, Solana Beach, CA, USA). Extraction was performed according to the manufacturer's instructions, and DNA samples were stored at – 20 ºC.

For each replicate site, five independent soil extractions were mixed together and used as a template for qPCR.

3.2.3 T-RFLP

Analysis was performed as described by Mendes and Tsai (2008). PCR was performed targeting the 16S rRNA Eubacteria gene, using primers 8F and 1492R (AMANN et al., 1995). The primer 8F was FAM labeled (LIU et al., 1997). The mix was composed of 1X Buffer, 25 mM dNTP, 100 mM of each primer, 1 U of Taq DNA polymerase to a final volume of 50 µl. The mix was submitted to one step of denaturation at 95º C for 5 min and 30 cycles of 95 C for 30 sec, 59 ºC for 45 sec and 72º C for 1 min, followed by a final extension of 72º C for 1 min.

The FAM labeled PCR amplicons were precipitated by the isopropanol method, adding 50 µl of H_2O and 60 µl of isopropanol to the samples, followed by centrifugation, discard of the supernatant, and repeating the same process with ethanol 80%, instead of water and isopropanol. The pellet was dried in a speed vac and resuspended in 20 µl of water. Amplicons were digested by the enzyme MspI. The mix was composed of 10 µl of the purified amplicons, 1.5 µl of buffer, 7 U of MspI, and water to a final volume of 15 µl. Reactions were incubated for 3 hours at 37°C and the digestion inactivated by heating at 68°C for 10 minutes. The digestion product was precipitated by adding 1M of sodium acetate and precipitating with 1 X absolute ethanol, supernatant discarded and pellet dried by a speed vac. DNA was resuspended in a mix of 9.75 µl formamide with 0.25 µl of the marker GeneScan 2500 Rox (Applied Biosciences), and loaded into an ABI-3100 automatic sequencer. The fragment sizes were analyzed by the GeneScan software and results exported to an excel .xls file for further statistical analysis.

3.2.4 qPCR analysis

Quantitative PCR was performed in an Opticon 2 machine (Bio-Rad) in 96-wellplates. All analyses were performed in duplicates. Each reaction was composed of 1 µl of the extracted DNA, 0.2 µl of the inhibitor resistant enzyme Klentaq, 0.5 ug of T4 Gene 32 Protein, to increase sensibility and reduce humic acid inhibition, 1X of KlenTaq buffer, 1 X of EvaGreen, 0.25 mM of dNTP, primers as described (Table 3.1) and molecular grade water to a final volume of 20 µl. The thermo cycler was programmed with an initial denaturation step at 95 ºC for 5 min followed by40 X 95 ºC for 15 s, annealing T for 30 s, 68 ºC for 30 s, followed by 5 min at 68 ºC, using annealing and reading temperature as described (Table 3.1). The melting curve was performed from 72 ºC to 95 ºC. The slope varied from -3.08 to -3.82 and standard curve r^2 from 0.990 to 0.999.

Standards were made from PCR amplifications of isolates or environmental DNA with each primer set. PCR products were cloned and 3-5 clones sequenced to provide specificity. We preferred to use PCR products dilutions as standards rather than plasmids, since plasmids can result in distortions of the quantification (HOU et al., 2010). Only *mcr*A and *pmo*A qPCRs were performed using plasmid standards.

Table 3.1 – qPCR Primers and amplification conditions for the different genes

Gene	Primer	Anealing (ºC)	Reading (ºC)	Reference
16S rRNA Bacteria	F563/BSR926	55	80	Claesson et al., 2010
*nos*Z	F/R	TD 65-62	83	Henry et al., 2006
pmoA	A189f/A682r	63	85	McDonald et al., 2008

3.2.5 Statistical analysis

The statistical design was completely randomized, with three replicate sites for each land use and five pseudo-replicates per site. For the analysis an average of the pseudo-replicates was used, to compare the three real replicates. The replicate sites were from the same topographic, edaphic and climatic conditions, assuming random

design. ANOVA analysis was performed and when significant effect was observed Duncan average comparison was performed ($p<0.05$). Soil chemical and gene data were analyzed by a Principal Component Analysis (PCA), using CANOCO (LEPS; SMILAUER, 2003).

3.3 Results

All data presented as an average of the three replicate sites.

3.3.1 Chemical analysis

Soil from the Cerrado and Forest sites showed lower pH, Ca and Mg than the agriculture and pasture sites (Table 3.2). Agriculture and Pasture showed generally higher values for the chemical data. Cation exchange capacity (CEC) was higher in Forest and the overall values among all the sites were correlated with the organic matter data (Pearson correlation, $r^2=0.78$, $p<0.0001$). Ammonium was higher in Forest and lower in soybean fields. Nitrate was higher in the soybean in Campo Verde, lower in the Cerrado and intermediate for the other samples. A higher ratio of nitrate to ammonium was observed for the soybean areas (Table 3.2).

Table 3.2 – Soil parameter for each land use (average of three replicate sites)

	Land Use	pH		M.O		P		S		K		Ca		Mg		CEC		NH_4		NO_3	
		$CaCl_2$		g dm^{-3}		- mg dm^{-3} -				------ $mmol_c$ dm^{-3} ------								--- mg kg^{-1} ---			
Forest	Forest	3.8	c	51	a	2	b	12	a	1	b	6	c	2	c	125	a	2.9	a	3.2	b
	Soybean	4.7	b	28	c	10	b	9	ab	1	b	18	b	5	bc	60	b	1.1	d	2.5	b
	Pasture	4.5	b	32	bc	2	b	5	b	1	b	13	b	7	ab	68	b	1.2	cd	0.7	c
Cerrado	Cerrado	3.8	c	49	a	2	b	7	ab	1	b	3	c	1	c	84	b	1.7	bc	0.2	c
	Soybean	5.2	a	45	a	55	a	7	ab	4	a	29	a	10	a	74	b	0.6	d	5.2	a
	Pasture	4.8	b	42	ab	10	b	4	b	2	b	17	b	9	ab	66	b	2.0	b	1.6	bc

* Values with the same letter in the column are not significantly different by the Duncan test ($p<0.05$).

Litter composition was different according to land use (Table 3.3). Forest and Cerrado presented higher lignin content, while soybean and pasture presented higher amount of easily degradable compounds such as carbohydrates and intermediate compounds such as cellulose. Forest presented higher fatty acids composition.

Table 3.3 – Litter composition of the different land uses (average of three replicate sites)

Sample	Hemicellulose	Cellulose	Lignin	Fatty Acids	CHOtt	C	N	C: N
	g.kg^{-1}	g.kg^{-1}	g.kg^{-1}	g.kg^{-1}	mg.kg^{-1}	%	%	
Forest	131.8 b	201.5 a	338.4 a	49.4 a	30.8 b	44.0 a	1.8 a	24.5 ab
Cerrado	45.3 c	168.1 a	316.4 a	19.6 b	24.7 b	39.7 a	1.3 b	34.2 a
Soybean	100.0 b	163.5 a	181.7 b	22.3 b	35.2 a	31.1 b	1.4 b	21.8 b
Pasture	255.2 a	234.3 a	184.8 b	8.5 c	30.3 ab	36.2 ab	1.2 b	31.7 a

* Values with the same letter in the column are not significantly different by the Duncan test ($p<0.05$).

3.3.2 Gene abundance

Distinct gene abundances were observed, with a trend of higher abundance in the Cerrado areas (Table 3.4, statistical analysis; Figure 3.1, standard variation).

Table 3.4 – Gene abundance in the different land uses (average of three replicate sites)

Sites	16S Bac	*pmo*A	*mcr*A	*nos*Z
	---------- (copies . g d.s. $^{-1}$) ----------			
Forest	2.E+09 b	3.E+08 cd	B.D.L.** b	8.E+06 ab
Soybean (Forest)	1.E+09 b	1.E+08 d	B.D.L. b	2.E+06 b
Brachiaria (Forest)	2.E+09 b	6.E+08 bc	B.D.L. b	6.E+06 ab
Cerrado	1.E+10 a	1.E+09 a	2.E+07 a	1.E+07 a
Soybean (Cerrado)	8.E+09 ab	4.E+08 cd	B.D.L. b	4.E+06 b
Brachiaria (Cerrado)	1.E+10 a	9.E+08 b	2.E+07 a	9.E+06 ab

* Values with the same letter in the column are not significantly different by the Duncan test ($p<0.05$).* *B.D.L., below detection limit (1E+03 . µl $^{-1}$ or 4E+05 . g d.s. $^{-1}$)

Cerrado areas presented higher abundances of Bacteria, and the Soybean area in the Cerrado region was similar to all sites of Sinop. For the gene *pmo*A, it was also observed in higher abundance in Cerrado areas. For the gene nosZ, Cerrado areas also have higher abundance in both biomes, however, areas with soybean presented lower gene abundance in general.

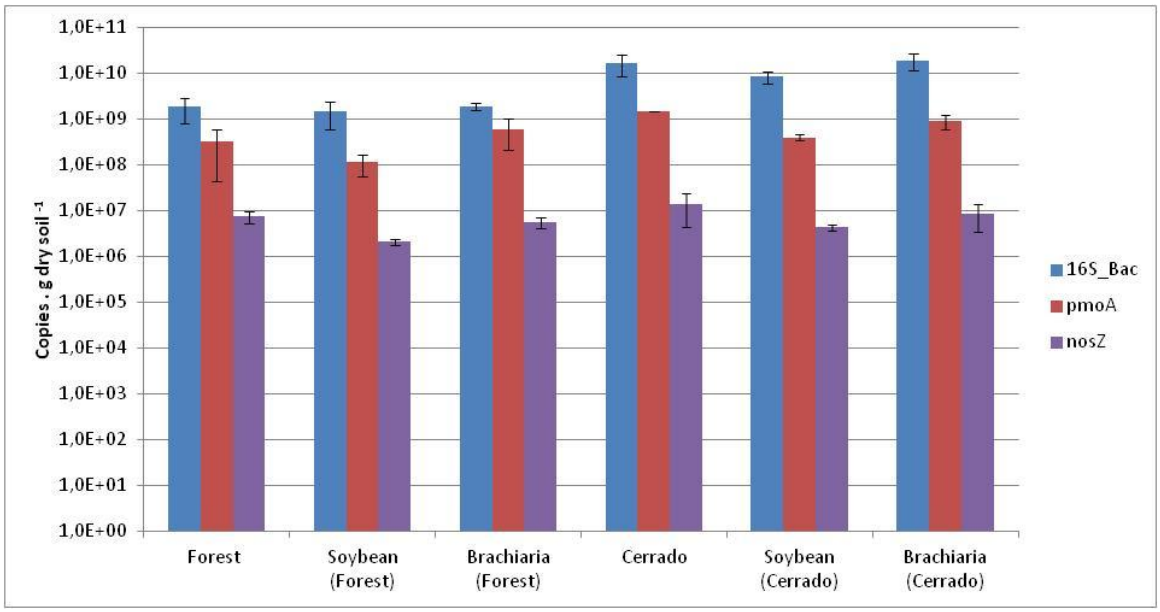

Figure 3.1 – Gene abundance in the different land uses (average of three replicate sites). Lines represent the standard deviation. Gene *mcr*A is not showed, since it was bellow detection limit for most of the samples

PCA analysis was performed comparing the different land uses with the soil and gene quantification variables (Figure 3.2).

A cluster can be observed in the bottom of the graph, which corresponds to samples of Cerrado and pasture from the same region. They are related to a higher abundance of the genes *pmo*A, *mcr*A and 16S rRNA from Bacteria, but not with chemical attributes of the soil. Two samples were separated from this cluster, one pasture sample that is in a central position and one Cerrado sample that is more related with ammonium on the right of the graph.

Another cluster can be observed in the right upper quadrant which corresponds to the soybean sites. They are related to higher chemical parameters, which are related to fertilizers and lime additions, P, K, pH, Ca, Mg and nitrate. In the Campo Verde areas it is more intense, and in the Sinop areas they are more moderate. Any data near the center presents moderate presence of the analyzed attributes.

Around the middle of the graph are most of the pasture samples, meaning that they present moderate presence of the attributes. As described before, most of the Campo Verde pasture samples were clustered with the Cerrado samples.

The other cluster is related with the Forest samples and higher abundance of organic matter, CEC, S and *nos*Z.

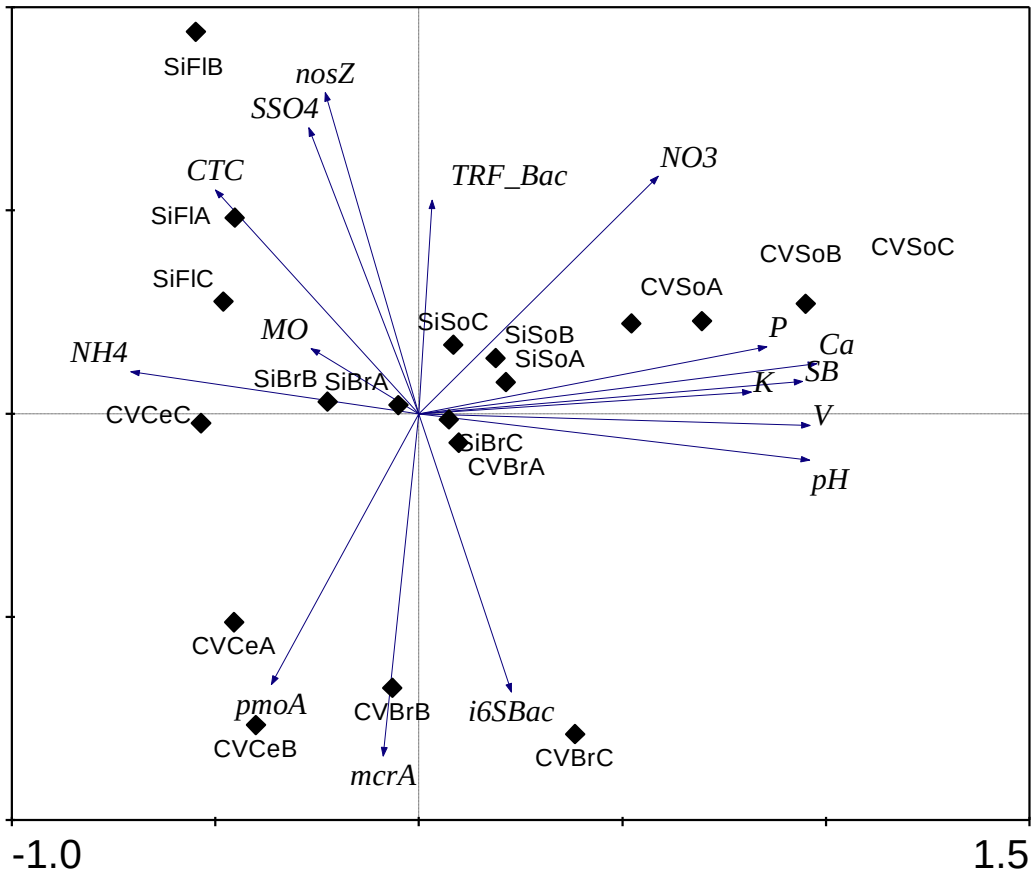

Figure 3.2 – Principal component analysis correlating the qPCR analysis with the soil chemical attributes. Small symbols represent the replicate sites for each land use, Si means Sinop, CV, Campo Verde, Fl, Forest, Ce, Cerrado, So, Soybean and Br, Brachiaria. The vectors represent the chemical attributes and the genes analyzed by qPCR (Table 3.2)

3.3.3 T-RFLP of the 16S rRNA bacterial gene

Several distinct patterns were observed among biomes and land uses in the Southwestern Amazon comparing the T-RFLP structure with the soil parameters data (Figure 3.3). But no statistical difference was observed comparing the average number of T-RFs (Table 3.5).

Several differences were observed when comparing the two main biomes, Tropical Forest and Cerrado, and the land converted to pasture and agriculture. Land use was the most important factor to distinguish the bacterial communities, and it was correlated with the soil chemical changes: pH - due to liming, and chemical fertility - due to fertilizer application.

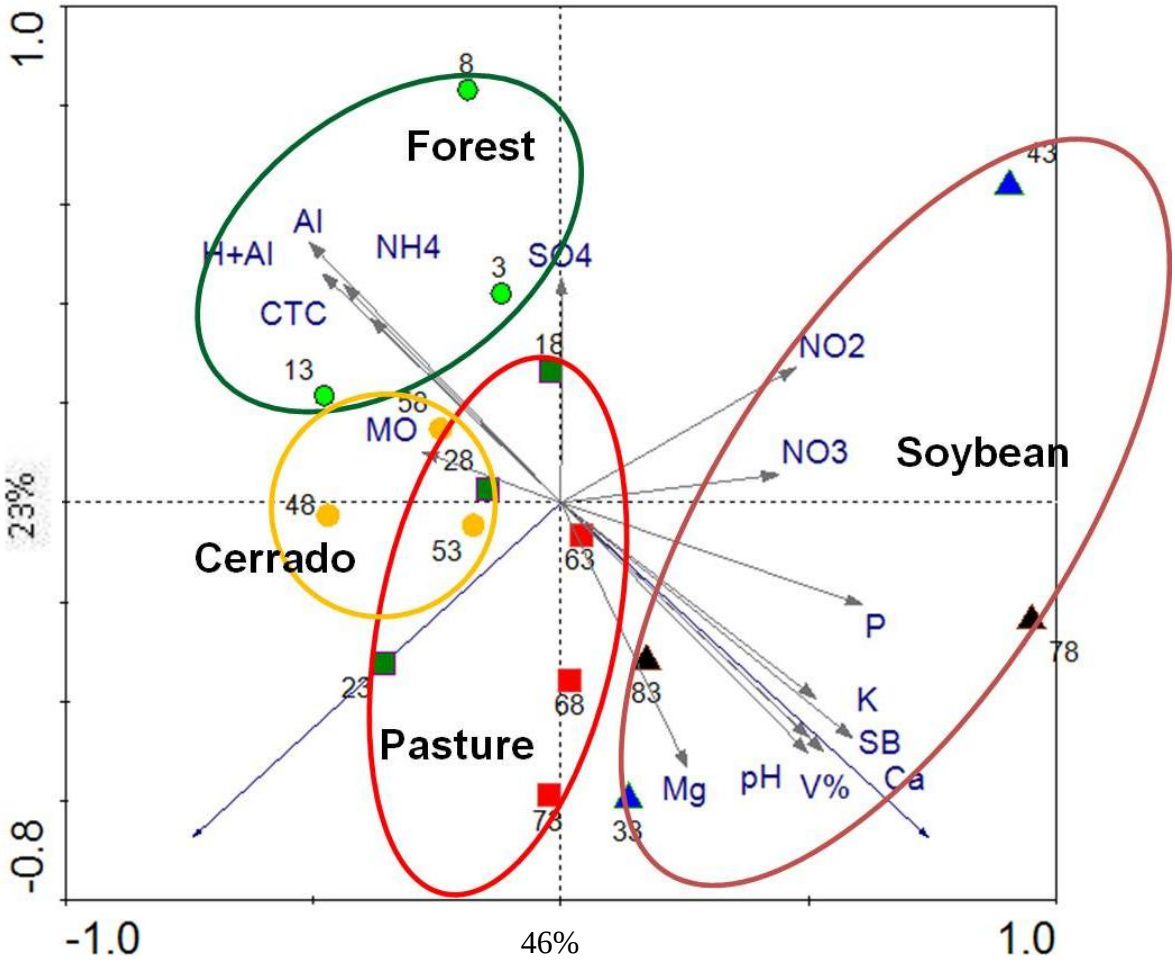

Figure 3.3 – Principal component analysis correlating the T-RFLP analysis with the soil chemical attributes. Small symbols represent the T-RFLP for each land use, circles are native vegetation, squares are pasture, triangles are soybean. Big circles delimit the land uses. Green color is Forest, yellow is Cerrado, red is pasture and purple is soybean. The vectors represent the chemical attributes (Table 3.2)

Table 3.5 – TRFs in the different land uses (average of three replicate sites)

Sites	16S Bac (TRFs . µl DNA)	
Forest	18	a
Soybean (Forest)	23	a
Brachiaria (Forest)	19	a
Cerrado	20	a
Soybean (Cerrado)	32	a
Brachiaria (Cerrado)	15	a

* Values with the same letter in the column are not significantly different by the Duncan test ($p<0.05$).

Pristine Tropical Forest and Cerrado formed distinct clusters, but they were more similar to each other than to pasture or soybean field.

Chemical parameters were the main drivers to separation of the samples and these parameters were correlated with the microbial structure data by T-RFLP. Since the land uses present distinct chemical attributes, it is not possible to separate if the microbial structure is correlated with the chemical data, or other characteristics of each land use, such as plant coverage, human interference, animal management in pasture, and agricultural practices in the soybean fields. To separate the effects would need a new study, comparing native vegetations on soils of different pH and nutrient levels with adjacent pasture and agricultural fields, also with pH and nutrient gradients.

3.4 Discussion

3.4.1 Chemical analysis

Liming and fertilizers were responsible for the most evident differences among the land uses and sites. Liming increased the pH, Ca and Mg of agriculture and pasture; consequently increasing sum of bases (BS) and bases saturation (V%). Since *Brachiaria* is tolerant to low pH and low fertility, farmers do not take the same care in this practice as in agriculture areas, were the economic benefits are evident. The higher fertility in the agriculture sites is clear, and agricultural management affected the soil parameters (CARVALHO et al., 2007).

Correlation analysis showed the relationship between OM and CEC, indicating importance of organic matter to the CEC (WILKE; LILIENFEIN, 2002). With land use change, a decrease in organic matter, and CEC was detected. The no till system adoption in the areas is relatively new, 10 years, and it is expected to increase organic matter in the next years, if adequate management will be taken, including erosion control and fertility maintenance (CARVALHO, 2010).

Differences were detected among the litter compositions (Table 3.3). It is clear that different vegetations produce different chemical composition. As reported, natural vegetation, present higher concentration of more recalcitrant molecules, such as lignin (MUNGAI; MOTAVALLI, 2006).

Litter composition is linked to microbial composition and accumulation of organic matter in the soil. In this study litter quantity was not quantified, but visually it was observed that there was a higher amount in the Forest and Cerrado areas. There are two reasons for the higher accumulation in the native areas, first is higher litter production, ranging from 0.6 to 0.8 $kg.m^2.year^{-1}$ in Amazon Forests (BRANDO et al., 2008), and second is the presence of more recalcitrant components, which take longer to decompose(Table 3.3).

3.4.2 Gene abundance

Different patterns were observed among the sites. In general, sites in the Cerrado biome present higher gene abundance them the Forest biome sites (Table 2.4). To the best of our knowledge, there are no other comparisons of gene abundance between Forest and Cerrado to discuss if this observation is a trend.

As well as difference in original vegetation there are also differences in geographic regions between sites. Climate is slight different, with lower precipitation in Cerrado areas in winter. A bigger spatial and temporal survey would need to be made to discuss if Cerrado areas have more bacteria than Forest areas, but this is a first insight.

Additionally, Bacterial 16S rRNA gene number copies vary from 1 to 14 copies per cell, depending on the species. Consequently, differences among areas need to be discussed with caution. Changes up to 14 times can be caused by community shifts, and not necessarily by abundance. For example, if in one area there is a predominance of bacteria with one copy of this gene and in another area bacteria

with 10 copies per cell, it can give a difference of 10 times in the result, as observed. As discussed in chapter 2, it will be assumed that copy number is the same in the different areas. It will be also assumed that microbial group abundance (cell numbers) is proportional to the related gene abundance (copy numbers).

Higher abundance of the microbial groups in native vegetation would be expected, since there is higher organic matter in the soil in these areas, and probably higher matter cycling.

Litter is also higher in the native areas. Even though litter is accumulated in the surface, fragmentation by the soil fauna and leaching may influence soil microbiota below ground (MEIER; BOWMAN, 2008). Both litter amount and composition can select for specific microbial communities and change C ratios of accumulation in soil. In the present study litter was surveyed as complementary information, but further studies should focus in experimental designs to address the specific influence of litter to the microbial community and C accumulation in the study areas (MUNGAI; MOTAVALLI, 2006).

The gene *mcr*A was detected only in Cerrado and pasture areas. Similarly, native areas and pasture presented higher *pmo*A amount, indicating higher methane production and consumption in these areas. Pasture usually represents high methane production, while native vegetation exhibits methane consumption (CARVALHO, 2010). The higher amount of these genes can be related with measurements of these processes.

It was surprising that *nos*Z abundance was lower in soybean areas, since it is expected to be an N rich environment. Soybean is related to nitrogen fixation, so most of the plant N comes from symbiosis, reducing the consumption of N from soil. Therefore, a higher availability of N in soil would be expected and consequently higher denitrifier abundance, which was not observed. Nitrate and ammonium are variable in relation to time therefore it is difficult to discuss *nos*Z correlation with these two nitrogen forms without a bigger survey in a longer period of time. A higher amount of *nos*Z in the Cerrado areas would be related to higher denitrification, explaining the lower nitrate level. But for this kind of assumptions, a bigger temporal survey should be made.

Multivariate analysis showed separation among areas. It showed a well defined cluster correlating soybean with liming and fertilization and also showed that pasture correlates to moderate chemical attributes. Both cluster according to our discussion about chemical data, liming and fertilization.

The other two clusters are more difficult to interpret. Investigating the Forest cluster, it had a higher correlation with CEC, *nos*Z, S and organic matter. Analyzing these parameters in detail, we detected a week correlation between *nos*Z and organic matter (0.40 $p<0.09$), which can an abundant substrate for denitrifier bacteria, supplying organic compounds and nitrogen to the processes. The correlation between organic matter and CEC was much higher, as described before, and also detected by the PCA analysis.

The other cluster, that included Cerrado and pasture samples, was related to *mcr*A, *pmo*A and the 16S rRNA Bacteria gene. In the PCA, information was concentrated in a graph of two dimensions, which results in loss of information. Since *mcr*A was only detected in Cerrado and pasture areas, it would be expected that the PCA analysis correlated this gene data and the site data. Other parameters are also related to these samples, as discussed for the univariate analysis. Higher bacteria abundance was detected for the Cerrado areas, and there was also a relationship between the metanogenic and metanotrophic genes.

In summary, the general trend for gene abundance was higher abundance detected in the Cerrado areas, indicating that microbial abundance and activity can be more intense in this biome than in the Forest or in the land use change to agriculture and pasture.

3.4.3 T-RFLP

The data provided several insights about how microbial communities change with land use change from Cerrado and Forest to agriculture and pasture. While no difference was detected among TRFs abundance among areas, several differences were found in the structure. These data is in accordance with other studies that showed diversity was not significantly affected, but structure changed with land use change.

In Western Amazon a T-RFLP analysis of the bacterial communities showed how community was influenced by soil attributes correlated to land use (JESUS et al., 2009). Community structure changed with pH and nutrient concentration. By DNA sequencing, bacterial communities presented clear differences among the different sites. Pasture and one of the crops presented the highest diversity. Secondary forest presented similar diversity with the community structure of the primary forest, showing that bacterial community can be restored after agricultural use of the soils.

Using ARISA, distinct microbial structures were also observed between agricultural and forest soils (NAVARRETE et al., 2010). Seasonal changes in the two different years of sampling and distinct band patterns were observed for the fungal, bacterial and archaeal richness.

Grossman et al. (2010) found differences between Anthrosols and Adjacent soils in the Amazon region. Anthrosols are agricultural soils used in the past by the native people in Amazon. Analysis of T-RFLP of the 16S rRNA genes provided clear distinction between the two types of soils, and the same result was observed using DGGE and 16S rRNA sequencing. While T-RFLP provided a good fingerprinting between Anthrosols and Adjacent soils, 16S rRNA sequencing provided better resolution of the changes, indicating *Verrucomicrobia* as an important group to the Anthrosols, *Proteobacteria* and *Cyanobacteria* as important to Adjacent soils; while *Pseudomonas*, *Acidobacteria* and *Flexibacter* were found in both sites.

These different techniques showed a high microbial diversity on Amazon soils. Fingerprinting techniques, such as T-RFLP and ARISA, were sensitive tools to detect difference in the microbial structure among the different sites and land uses. However, DNA sequencing provided a better resolution of the diversity, i.e. identify taxonomic groups and report unknown *Bacteria* that may belong to new taxonomic groups.

These pioneer studies showed, in general, that diversity does not decrease from pristine vegetation to agricultural uses, but the structure of microbial community as a whole is affected by land use changes. The same trend was observed in the present study.

3.5 Conclusions

Chemical changes were detected due to land use. Agriculture and pasture increased the chemical attributes of the soil, mainly due to liming and fertilization.

Using qPCR and T-RFLP approaches, it was clear that land use changes significantly influenced the structure of microbial communities in the Amazonian soils. Further use of sequencing may help to show in detail the differences.

A PCA analysis showed correlation among the soil chemical and the biological attributes, showing that microbial structure responded to the environmental changes caused by land use change.

In general, the Cerrado areas presented higher gene abundance, indicative of higher microbial abundance and activity in these sites.

4. LAND USE CHANGE IN THE CERRADO: CHANGES IN C, N AND MICROBIAL ATTRIBUTES

In general, land use changes in Brazil do not occur only in tropical forests. Instead, they happen mainly in the Cerrado, or “Brazilian savanna”, yet this is hardly the focus of research. This chapter reports a case study of how land use change from Cerrado to agriculture and pasture affected certain factors, such as soil chemical characteristics and microbial parameters. For example, when land use changed from Cerrado to agriculture and pasture, Nitrogen concentrations in the soil were affected. This change may be attributed to management practices since manure, soybean N fixation, and fertilization in successive crops added N to the soil. Liming and fertilizers were land management practices that also changed other chemical parameters such as P, Ca, Mg, and K concentrations, and soil pH. The abundance of Archaea and fungi correlated with change in land use, but biomass did not. Although these results suggest that further studies will be needed, we can already conclude that soil chemistry and microbiota change with land use change.

Key words: Cerrado. Land use change. Soil parameters.

4.1 Introduction

Despite alarms about Amazon Forest deforestation, most of the agriculture expansion in Brazil occurs over the Cerrado biome. Following deforestation, not only the biomass but also the soil C and N stocks is mobilized and partly emitted in the form of the greenhouse gases (GHG) CO_2, CH_4 and N_2O.

However, losses of soil C and N stocks are greatly modified by management. Under specific management practices applied to tropical soils, soil organic matter (SOM) may increase, which is called C sequestration. Conversely, SOM is reduced when inadequate practices such as excessive tilling or pasture degradation is applied (CARVALHO et al., 2007; CARVALHO et al., 2010). Increases in SOM are not only important from the viewpoint of GHG production, but also due to its positive effects on soil fertility, Cation Exchange Capacity (CEC), nutrient cycling, soil structure, erosion protection and water retention (CARVALHO et al., 2010, BAYER et al., 1999).

The soil microbial community is essential for the formation and decomposition of SOM. Differences in soil microbial biomass and microbial community structure have been found to be good indicators for site specific SOM matter dynamics and thus for soil fertility (FRAZÃO et al., 2010; FRAZÃO et al., 2011).

Even though it is well established that land use changes significantly affect microbial biomass, as well as microbial community structure, changing the abundance of Bacteria, Fungi and Archaea, information about the effects of land use change in Cerrado regions on the microbiota is scarce.

In this study, we investigated how land use change from Cerrado to pasture (*Brachiaria*) or agriculture (soybean-corn/cotton) has affected soil C, N, chemical fertility, microbial biomass and the abundance of *Bacteria*, *Archaea* and *Fungi*.

4.2 Material and Methods

4.2.1 Survey

Survey was conducted in the Marabá Farm, Campo Verde, MT (Chapter 1). Soil samples were taken from the 3 different land uses (pasture, agriculture and Cerrado), in three replicate sites of 1 ha for each land use. At each site, 10 plots

were randomly selected and individual soil samples were taken from 0-5, 5-10, 10-20 and 20-40 cm for analysis.

4.2.2 Soil C and N

Soil samples were first air dried and sieved at 2 mm. Then 10 g of soil was ground and sieved to 0.25 mm for determination of total C and N contents, which were analyzed by total combustion (CARVALHO et al., 2007).

Bulk density was determined through soil sampling in volumetric rings. The weight was determined by drying overnight at 60ºC. The density was determined by dividing the dry weight by the ring volume.

C and N stocks were calculated multiplying the C concentration by the bulk density of each individual layers, and converting the values for Mg per ha.

4.2.3 Chemical analysis

Soil samples were processed as described by CARVALHO et al. (2007). Soil samples were air dried and sieved at 2 mm. Soil pH was measured in 0.01 M $CaCl_2$. Organic Matter (OM) was determined by dichromate digestion following a back-titration with $(NH4)_2Fe(SO4)_2$. The concentrations of P, Ca, Mg, S and K were determined by using ion exchange resins and analyzed according to Cantarella and Quaggio (2001).

4.2.4 Microbial biomass

The soil microbial biomass carbon (MBC) and the soil microbial biomass nitrogen (MBN) were determined by using the fumigation-extraction method (VANCE et al., 1987). For this analysis, fumigated and non-fumigated aliquots of 25 g of soil samples were extracted with 0.5 M K_2SO_4. Organic C in the sulfate extracts was oxidized by acid dichromate digestion. The excess dichromate was determined by back-titration with $(NH4)_2Fe(SO4)_2$. MBC was calculated as the difference between organic C content in fumigated and non-fumigated soils using kEC of 0.33 (FEIGL et al., 1995). Microbial nitrogen (NMIC) was determined by the ninhydrin reactive

compound quantification method using the conversion factor kEN = 0.65 (SPARLING et al., 1993).

4.2.5 qPCR analysis

Quantitative PCR (qPCR) was performed by QuantiFast mix for the genes 16S rRNA Eubacteria, using primers 16SEF and 16SER (BACH, 2002), submitted to one step of denaturation 95º C for 5 min and 35 cycles of 95 ºC for 15 sec and 60º C for 1 min; and for 18S rRNA Fungi, primers EF4f and Fung 5r (VAN ELSAS, 2000), were performed with one step of denaturation 95º C for 5 min and 40 cycles of 95º C for 15 sec, 61º C for 15 sec. QTech mix was used for the gene 16S rRNA Archaea using primers 915F and 1059R (YU, 2005) at 40 cycles of 94º C for 15 sec, 63º C for 30 sec and 72º C for 1 min. The reaction mix were made according to the manufacturer's instructions, diluting the qPCR mix 2X, adding 100 mM of the primers and using molecular grade water to a final volume of 25 µl per reaction.

Standards were made from amplicons of isolates or environmental DNA with each primer set. Amplicons were cloned in *E. coli* and 3-5 clones sequenced for verification. Plasmids were quantified using a spectrophotometer, concentrations were calculated and dilutions for each specific gene were used as standard curves for qPCR.

4.2.6 Statistical analysis

The sampling design was completely randomized, with three replicate sites for each land use studied, and ten pseudo-replicates per replicate site. In the results section, each replicate site is alphabetically labeled (A, B, or C). All three replicate sites were from the same farms under the same management. The replicates sites were surveyed to show spatial variability within the land uses. The area was located within similar topography, edaphic and climatic conditions, assuming random design.

Obtained data were analyzed using analysis of variance (ANOVA) and the averages compared through Duncan's test ($p<0.05$ for the chemical data and $p<0.1$ for the biological data). Pearson correlation coefficients were used to assess the relationships among variables.

4.3 Results

4.3.1 Soil C and N

Differences in total carbon content were not detected among land uses when considering the entire layer (0-40 cm), but statistical differences were detected when considered individual layers (Duncan, p<0.05). Total C was higher in the upper layer, declining with soil depth (Figure 4.1). Comparing the three land uses, total C was significantly higher in the layer 0-5 cm of Cerrado, and lower in Cerrado at 10-20 cm. No difference in the others depths was observed (Duncan p<0.05).

For nitrogen content, differences among land uses were detected when considering the entire soil layer (0-40cm). Nitrogen content was higher in the agriculture and pasture sites compared to Cerrado. Nitrogen content declined with soil depth (Figure 4.2). N content was equal for the land uses in the 0-5 cm layer, higher in agriculture and pasture for the 5-10 cm and 10-20 cm depth, and higher for Cerrado and pasture in the 20-40 cm depth (Duncan, p<0.05).

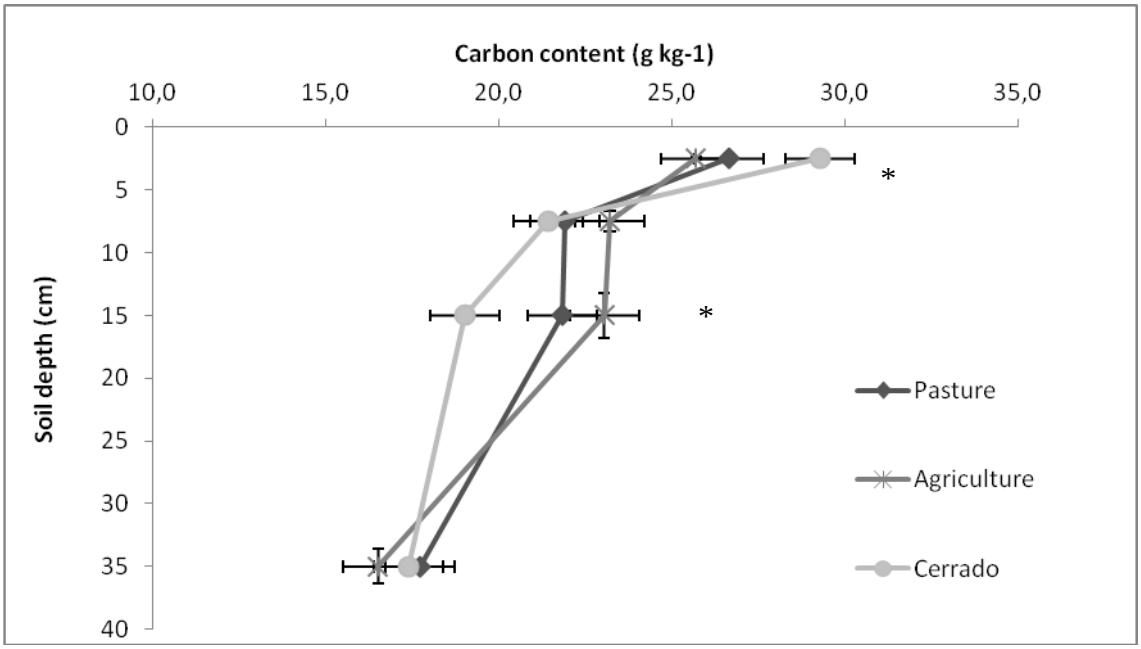

Figure 4.1 – C content in different soil depth of the different land uses. Each point is an average of the three replicate sites and the error bars correspond to the standard deviation. The symbol ” * “ indicates when significant statistical difference was detected (Duncan, p <0.05)

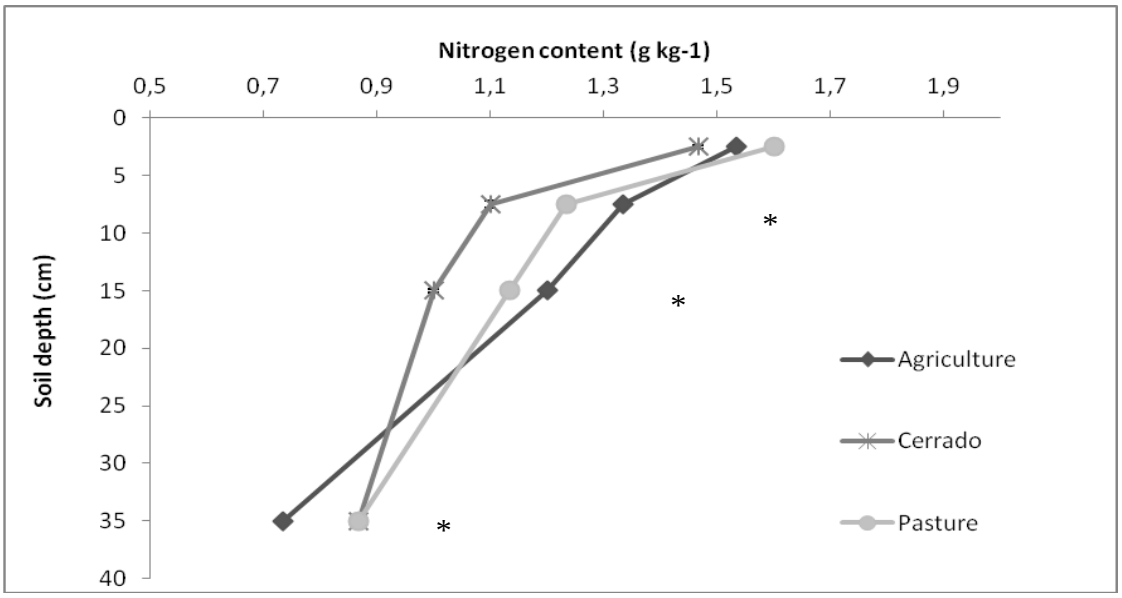

Figure 4.2 – N content in different soil depth of the different land uses. Each point is an average of the three replicate sites and the error bars correspond to the standard deviation. The symbol ” * “ indicates when significant statistical difference was detected (Duncan, p<0.05)

Bulk density varied from 1.05 to 1.19 in the Agriculture and Pasture areas, and it was significantly higher than the Cerrado area, that varied from 0.89 to 0.99 (Duncan, p <0.05). The lower values in both areas correspond to the 20-40 cm layer.

Differences in C stocks were detected among land uses, considering the 0-40 cm layer (Table 4.1). Pasture presented the highest values of C stocks while Cerrado contained the lowest. No clear differences were observed in the superficial layers (0-5 cm and 5-10 cm). In the 10-20 cm layer, Cerrado had lower values, but no difference was observed between agriculture and pasture. At the 20-40 cm depth, C stocks were highest in the pasture.

Table 4.1 – C stocks in different soil depth of the different land uses

	C (Mg.ha-1)									
Land use	**0-40 cm**		**0-5 cm**		**5-10 cm**		**10-20 cm**		**20-40 cm**	
Pasture A*	95.8	a	16.8	a	12.8	ab	24.1	a	42.2	a
Pasture B	95.3	a	16.0	a	12.8	ab	24.3	a	42.2	a
Pasture C	91.6	ab	14.6	ab	13.0	a	24.0	a	40.1	a
Agriculture A	81.3	b	13.3	ab	12.9	ab	23.9	a	31.2	b
Agriculture B	90.2	ab	13.8	ab	13.9	a	26.5	a	36.0	ab
Agriculture C	93.5	ab	15.7	a	13.6	a	27.9	a	36.4	ab
Cerrado A	75.6	c	14.8	ab	12.5	ab	18.0	b	30.3	b
Cerrado B	71.2	c	11.1	b	10.9	b	16.9	b	32.3	b
Cerrado C	76.7	c	16.3	a	11.8	ab	18.8	b	29.8	b

* The different letters in each land (A, B and C) use refer to the different replicate sites, all sites were surveyed to test spatial variability within the land uses.
** Values with the same letter in the column are not (Duncan, p <0.05).

Differences in N stocks were detected among land uses (Table 4.2). Again, N concentration in the pasture was significant higher in the 0-40 cm layer, followed by agriculture and the Cerrado area. No clear significant differences were observed in the layer 0-5 cm, 5-10 cm and 10-20 cm, but a trend of lower values in Cerrado was observed. N stock was higher in 20-40 cm in the pasture, but no difference between Agriculture and Cerrado was observed (Table 4.2).

Table 4.2 – N stocks in different soil depth of the different land uses

	N (Mg.ha^{-1})									
Land use	**0-40 cm**		**0-5 cm**		**5-10 cm**		**10-20 cm**		**20-40 cm**	
Pasture A*	5.1	a	1.0	a	0.7	a	1.2	ab	2.1	a
Pasture B	4.7	ab	0.9	a	0.7	a	1.2	ab	1.9	a
Pasture C	5.1	a	0.9	a	0.8	a	1.3	a	2.1	a
Agriculture A	4.3	b	0.8	a	0.8	a	1.2	ab	1.5	b
Agriculture B	4.4	b	0.8	a	0.8	a	1.4	a	1.5	b
Agriculture C	4.8	ab	0.9	a	0.8	a	1.5	a	1.7	b
Cerrado A	3.7	c	0.7	ab	0.7	a	0.9	b	1.4	b
Cerrado B	3.6	c	0.6	b	0.5	b	0.8	b	1.6	b
Cerrado C	4.1	b	0.8	a	0.6	ab	1.0	ab	1.6	b

* The different letters in each land (A, B and C) use refer to the different replicate sites, all sites were surveyed to test spatial variability within the land uses.
** Values with the same letter in the column are not different (Duncan, p <0.05).

4.3.2 Soil chemical parameters

Cerrado showed lower pH, P, K, Ca and Mg values than the agriculture and pasture sites. Variability within different sites of the same land use was detected (Table 4.3).

Table 4.3 – Chemical analysis of the different land uses (0-10 cm soil depth)

Land use	PH ($CaCl_2$)	OM ($g.kg^{-1}$)	P ($mg.dm^{-3}$)	S ($mg.dm^{-3}$)	K ($cmol_c.dm^{-3}$)	Ca ($cmol_c.dm^{-3}$)	Mg ($cmol_c.dm^{-3}$)	CEC ($cmol_c.dm^{-3}$)
Pasture A	4.7 c*	38.0 b	5.7 d	3.9 b	1.3 def	17.0 c	6.1 bc	70.5 cd
Pasture B	4.6 c	46.3 b	2.6 d	3.1 b	2.0 cd	11.0 d	5.0 cd	60.1 d
Pasture C	5.2 ab	41.9 b	47.7 bc	4.1 ab	2.6 bcd	23.6 ab	13.7 a	68.2 cd
Agriculture A	5.1 b	45.0 b	26.6 c	4.3 ab	4.0 ab	21.4 bc	9.4 ab	66.8 cd
Agriculture B	5.2 ab	44.0 b	66.3 ab	7.6 a	3.7 ab	25.0 ab	9.9 ab	74.1 bc
Agriculture C	5.4 a	52.1 a	101.3 a	5.3 ab	4.5 a	29.9 a	10.0 ab	79.9 ab
Cerrado A	3.8 d	52.6 a	2.1 d	3.9 b	0.8 f	1.1 d	1.0 d	86.7 a
Cerrado B	3.9 d	53.1 a	2.3 d	3.9 b	1.0 ef	2.9 d	1.4 d	83.3 ab
Cerrado C	3.8 d	52.7 a	2.3 d	5.7 ab	0.8 f	1.0 d	1.0 d	85.1 a

* Values with the same letter in the column are not different by Duncan test ($p<0.05$).

4.3.3 Microbial biomass

A great variability was detected in microbial biomass in the different replicate sites (Table 4.4).

Table 4.4 – Microbial Biomass, C and N in different land uses (0-10 cm soil depth)

Land use	Plot	MBC ($ug.g^{-1}$ soil)	MBN ($ug.g^{-1}$ soil)	MBC: MBN	C ($g.kg^{-1}$)	N ($g.kg^{-1}$)	C:N
Pasture	A	730.1 de*	85.2 a	8.6	25.6 a	1.5 a	17.1 a
Pasture	B	797.0 e	105.1 a	7.6	24.0 a	1.3 abc	18.5 ab
Pasture	C	888.0 cd	104.1 a	8.5	23.2 a	1.4 abc	16.6 b
Agriculture	A	625.3 ef	47.9 b	13.1	24.0 a	1.4 ab	17.1 b
Agriculture	B	314.9 g	37.9 b	8.3	24.7 a	1.4 ab	17.6 b
Agriculture	C	1371.9 a	51.4 b	26.7	24.6 a	1.4 ab	17.6 b
Cerrado	A	1154.0 ab	82.2 a	14.0	24.4 a	1.2 bc	20.3 a
Cerrado	B	899.8 cd	80.4 a	11.2	23.5 a	1.2 c	19.6 a
Cerrado	C	1054.0 bc	83.0 b	12.7	24.6 a	1.3 abc	18.9 ab

* Values with the same letter in the column are not different by Duncan test ($p<0.1$).

Higher MBC in Cerrado areas compared to pasture and agriculture was observed. MBN was clearly higher in Cerrado and pasture than the agriculture. Total C and N did not differ in this layer, but a higher ratio of C:N was observed in the Cerrado sites.

A correlation analysis was performed for all data and relevant results presented in Table 4.5. No correlation was found between NMB and the other attributes, so data was not shown in the table.

Table 4.5 – Pearson correlation coefficient (r) between selected parameters

Parameters	All data	Pasture	Agriculture	Cerrado
C X CEC	0.40 **	NS	0.41 *	NS
C X N	**0.79 *** **	**0.94 *** **	**0.90 ** **	NS
C X OM	0.46 ***	0.41 *	NS	**0.91 ** **
C X B (Ca, Mg and K)	NS	NS	NS	**0.93** **
C X P	NS	NS	NS	**0.87 ** **
MBC X pH	NS	**0.77 *** **	NS	NS
MBC X C	NS	0.23 ^{ns}	NS	NS
MBC X OM	**0.54 *** **	**0.52 ** **	**0.73 ** **	NS
MBC X C:N	0.36 **	**-0.50 ***	NS	NS
MBC X MBN	NS	NS	NS	**-0.86 ** **

NS, Not significant, * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

No correlation was observed between MBC and soil C, however MBC and OM were correlated in the pasture and agriculture sites. Correlation between MBC and pH and CMB was observed for the pasture site while MBC and MBN were correlated for the Cerrado. A negative correlation between CMB and C:N was observed for the pasture as well as for MBC and NMB for the Cerrado area.

Taking all data into account, correlation between C and CEC, C and N, C and OM, MBC and OM, and MBC and C:N were detected.

4.3.4 Abundance of bacteria, archaea and fungi

Bacteria abundance was higher in the pasture site, but no difference was observed between the different land uses and the other plots. Higher *Fungi* abundance in *Cerrado* than the other plots was observed and higher *Archaea* abundance in the agriculture sites was also observed (Table 4.6).

Table 4.6 – Gene copies of *16S rRNA* (Bacteria), *16S rRNA* (Archaea) and *18S rRNA* (Fungi) in the different land uses (copies.mg^{-1} of soil)

Land use	Plot	*16S rRNA Bacteria* (copies.g^{-1} soil)		*16S rRNA Archaea* (copies.g^{-1} soil)		*18S rRNA Fungi* (copies.g^{-1} soil)	
Pasture	A	2.41E+10	a	4.87E+06	bc	2.29E+07	a
Pasture	B	7.57E+09	b	6.98E+05	c	6.04E+06	bc
Pasture	C	1.50E+10	ab	1.93E+06	c	7.45E+06	bc
Agriculture	A	7.25E+09	b	6.21E+06	bc	3.05E+06	c
Agriculture	B	7.02E+09	b	3.08E+06	bc	1.94E+06	c
Agriculture	C	7.43E+09	b	1.28E+07	a	5.99E+06	bc
Cerrado	A	1.50E+10	ab	3.70E+06	bc	1.41E+07	ab
Cerrado	B	1.28E+10	b	1.18E+06	c	2.10E+07	a
Cerrado	C	1.27E+10	b	7.91E+06	ab	1.39E+07	ab

* Values with the same letter in the column are not different by Duncan test (p<0.1).

Correlation was detected between gene abundance for the ribosomal genes of *Bacteria*, *Archaea* and *Fungi* (Table 4.7). Correlation was detected between *Bacteria* and *Archaea* in pasture, for *Bacteria* and *Fungi* in Cerrado, and for *Archaea* and *Fungi* in pasture and agriculture.

Table 4.7 – Pearson correlation coefficient between selected parameters

Parameter	All data	Pasture	Agriculture	Cerrado
Bacteria X Archaea	NS	0.47 *	NS	NS
Bacteria X Fungi	0.37 **	NS	NS	**0.87 *** **
Archaea X Fungi	NS	**0.68 ** **	**0.76 *** **	NS

NS, Not significant, * (p<0.05), ** (p<0.01), *** (p<0.001).

The gene abundance analyses were performed during the interchange in Germany, but only for the 0-5 cm layer. Because of this, no correlation analysis was performed between gene abundance and the other parameters, since they were analyzed in different depths.

4.4 Discussion

4.4.1 Soil C and N

It is well known that conventional tillage may lead to further reduction of soil C content in the topsoil. Therefore, our finding that C content was not significantly affected by land use may be due to the introduction of no-tillage systems across the region approximately 10 years ago. We hypothesize that soil C was lost in the first years after conversion into arable fields, but the introduction of conservational practices replenished the soil C pool thereafter.

In the agriculture sites, increase in soil N concentration is a result of soybean nitrogen fixation, and fertilization of the succession crops. In the pasture, higher N concentration may be due to animal feces and urine (CARVALHO et al., 2007).

For the C and N stocks, we observed an increase in pasture and in agriculture sites. This trend may be attributed to greater bulk density due to compacting C and N in the soil layers. The calculated C stocks are in the range of previously studies in the Cerrado, presented in the review of Batlle-Bayer et al. (2010). Only a few studies have investigated soil characteristics at a depth range of 0-20 cm. The Cerrado C stocks presented in this work at 74.5 Mg $C.ha^{-1}$ were in the range of other studies at 61.1-81.9 Mg C. ha^{-1} taken at a depth range of 0- 40 cm (D'ANDREA et al., 2004; FREITAS, 2000). However, the pasture site had a higher value (97.8 Mg $C.ha^{-1}$) than previously found values of 40.7 to 74.2 Mg $C.ha^{-1}$, presented by the same authors. For the agricultural sites, the value of 88.3 Mg $C.ha^{-1}$ was in range at 61.1 to 84. 4 Mg $C.ha^{-1}$.

Even though increases in soil N stocks are positive for agriculture, the environmental consequences (e.g. N_2O losses to the atmosphere or nitrate leaching) have not been studied for these sites.

4.4.2 Soil chemical parameters

Liming and fertilizers were responsible for the most evident differences among the land uses and sites. Liming increased the pH and BS% of agriculture and pasture. Since *Brachiaria* is tolerant to low pH and low fertility, farmers do not use the same practices as in agriculture areas, where the economic benefits are evident.

So, it is clear the higher fertility in the agriculture sites, consequence of the agricultural management that affected the soil parameters (CARVALHO et al., 2007).

For the Cerrado areas the importance of the C for fertility is clear because the correlation analysis shows a positive relationship between C, BS% and P, indicating nutrient cycling (WILKE; LILIENFEIN, 2002).

4.4.3 Microbial biomass

A high variability was observed in the results, which usually occurs in this type of analysis (SIQUEIRA et al., 2010; FRAZÃO et al., 2010). Despite the high variability of the result, which consequently resulted in variable statistical results, it is possible to observe some trends. In general, a higher microbial biomass in preserved and natural soils was observed in this study in accordance with previous work (SIQUEIRA et al., 2010; FRAZÃO et al., 2010).

According to Wallander et al. (2003), variations in C:N ratios of soil biomass have often been attributed to differing properties of fungal and bacterial biomass since fungi are reported to have a higher C:N ratio (5–15) than bacteria (3–6). Thus, higher CBM:NBM may indicate a higher abundance of Fungi, which is a trend that was observed for the Cerrado and agriculture sites. These MBC:MBN ratios are in agreement with the qPCR analysis, that showed a trend of higher Fungi in Cerrado. In pasture was observed a lower MBC:MBN ratio, which is in accordance with the trend observed by qPCR.

4.4.4 Abundance of bacteria, archaea e fungi

A higher abundance of Fungi and Bacteria in the Cerrado and Pasture areas was detected. The abundance of *Archaea* seems to be a good indicator of land use change (NAVARRETE et al., 2010) as it is related to sites where the soil had significant chemical changes.

Some trends were observed in both, MBC:MBN and qPCR results, the higher abundance of Fungi in Cerrado and Bacteria in Pasture. The Cerrado litter has a higher C:N ratio with a more complex material than agriculture and pasture sites

(Chapter 3). These litter characteristics can explain the bigger contribution of Fungi in these areas; because Fungi are known as better decomposer of more complex organic compounds, such as lignin.

One of the soybean sites presented a higher biomass value and Fungi abundance. We did not quantify litter amount, but it is possible that this replicate site may contain higher litter than the other sites. Another hypothesis is that this site received less fungicide. The higher values of MBC and fungi can be related to the decomposition of the leaves, presenting lower C:N ratio, and higher decomposition rates (TORRES et al., 2005).

4.5 Conclusions

Land use change from Cerrado to agriculture and pasture did not affect the C concentration of the 0-40 cm, but changed the C stocks. C stocks were higher in pasture and agriculture than Cerrado. The higher bulk density of these soils increased the C amount in the soil layers, and consequent C stocks.

Nitrogen concentrations and stocks were higher in pasture and agriculture sites than in the Cerrado. The difference was a consequence of management type since manure in the pasture; soybean N fixation and fertilization in the succession crops contain higher N content.

Liming and fertilizers in agriculture and pasture changed chemical parameters of the soil, especially P, bases (Ca, Mg and K) and pH. In Cerrado, OM is important for soil fertility, especially cycling bases and P.

Biomass presented higher variability, but a trend of higher amount in Cerrado and pasture was observed. Litter is more complex in Cerrado and *Fungi* is a more abundant group in these soils, while the abundance of *Archaea* seems to be an indicator of land use change to pasture and agriculture.

5. INFLUENCE OF *G. max* AND *B. brizantha* ON FOREST AND CERRADO SOILS

In the previous chapters it was not possible to separate the influence of a changing vegetation cover from the general effects of land use change on the microbial community and the soil chemical parameters. Land use change modified several soil attributes in parallel, rending any conclusions of the effect of changes in plant cover on soil processes speculation. However, under mesocosms conditions, environmental interference can be reduced enough so that the effects of plants on the microbial community can be addressed. The aim of this chapter was to study the effect of Brachiaria and soybean planting on the abundance of microbial groups related to the C and N cycles, and to correlate this change in abundance of soil bacterial groups to soil parameters, namely pH, C, N, and the flux of the gases carbon dioxide, methane, and nitrous oxide. Soil mesocosms of Forest and Cerrado were assembled, and Brachiaria or Soybean was planted. A higher abundance of Bacteria in mesocosms with plants compared to control mesocosms without plants was observed. The gene *nos*Z was correlated with C and N, and consequently, with a higher presence of denitrifier bacteria in Cerrado soils. Due to technical problems, we could not analyze all pseudo-replications individually at this time. It is highly likely that the individual analysis of pseudo-replicates, the analysis of additional genes and the use of diversity tools, such as sequencing, will provide better correlations and insights in the influence of these plants in the soils.

Key words: Mesocosms. Rhizosphere. Native soils.

5.1 Introduction

Field experiments may show high variability due to varying soil, climate and environmental conditions of the study area. This variability may affect the interpretation of results or even derail the testing of the hypothesis in question. To minimize these problems, many researchers use the incubation of soil with appropriate treatment, under controlled conditions, calling them microcosms or mesocosms. Generally the term of microcosm is used to bench scale experiments, since we use bigger amounts of soil in greenhouse we opted for the term mesocosm. Using this technique, structural changes in soil microbial communities have been studied the (SUBBARAO et al., 2009; BREMER et al., 2007; BRAKER et al., 2003).

In Chapter 2 and 3, it was not possible to separate plant effect from land use effect on microbial community and soil parameters. A change in land use modifies several soil attributes at same time, making speculative any attempt to address the plants' effect on the processes. In the previous chapters the effect of land use in the soil chemical parameters was clear and in chapters 4 and 5 the goal was to address plant effect on the microbial communities.

Recently, Bremer et al. (2007), using microcosms, showed that the denitrifying community is affected by the rhizosphere of plants. The plant effects include nutrient and water uptake from the soil, gas exchange, especially root respiration, and root exudates. Regarding root exudates, this includes from beneficial effects, as organic compounds that stimulate microbes growth (BREMER et al., 2007), to suppressive effects, as molecules that inhibit microbial processes, such as denitrification (SUBBARAO et al., 2009).

The aim of this chapter was to study the effects of brachiaria and soybean in the abundance of microbial groups related to the C and N cycles and to correlate this to soil parameters, such as pH, C, N, and to the flux of the gases carbon dioxide, methane and nitrous oxide.

5.2 Material and Methods

5.2.1 Survey

The survey was carried out in Sinop and Campo Verde, MT, as described in chapter 2. Soil samples were surveyed from the Forest and Cerrado areas, in three replicate sites and three pseudo replicates per replicate site.

Trying to maintain soil structure, and consequently the anaerobic micro sites in the samples, a maximum undisturbed possible sample was made. PVC tubes of 40 cm X 15 cm were used, and small holes were made in the upper part of each tube, allowing the insertion of an iron rod to pull out the tubes from the soil. The tubes were introduced into the soil with the help of a block of wood and mallet and then pulled out with the iron rod. (Figure 5.1).

Three tubes were surveyed per pseudo replicate, for use in the greenhouse experiment. In one of these tubes Brachiaria was sowed, in another soybean and a third tube was kept as control without plants. So in total two biomes were surveyed: Forest and Cerrado, in three replicate sites, in three pseudo replicates, with three tubes per pseudo replicate, a total of 54 tubes.

Since it was not possible to introduce the tubes up to the end, because of the space needed to insert the rod to pull them out, a head space over the soil was kept. Because of this head space inside the tubes, a 0-30 cm soil layer was considered per tube.

For the Cerrado area it was not possible to survey as not disturbed. The soil was too hard to allow the penetration of the tubes. As an alternative, soil was dug in each pseudo replicate site and separated in two piles, one of 0-10 and other 10-30 cm in depth. After this, each tube was individually assembled, first soil from the 10-30 cm layer was added and then from the 0-10 cm layer, trying to keep the structure as close and similar to the original soil profile as possible.

These tubes were transported in the back of a pickup truck, in the vertical position, carefully wrapped in bubble wrap in order to cause less disturbance to the samples. To avoid large temperature fluctuations during transport, which could lead to microbial changes, the tubes were covered by styrofoam boxes.

The temperature was monitored in the back of the pickup truck with a maximum and minimum thermometer, ranging between 20 and 36°C, within the soil's natural temperature oscillation range.

Picture 5.1 – Soil sampling, introduction of the tube with a piece of wood (A) and mallet (B) and removal of the tube with the aid of an iron rod (C and D)

5.2.2 Experiment at greenhouse

The experiment was conducted in a greenhouse at CENA/USP using the samples collected in the tubes previously described (15 cm diameter, 30 cm of soil collected). As described before, 27 tubes for Cerrado and 27 tubes for Forest

were surveyed. Brachiaria was sown in nine tubes, Soybean in nine other tubes and the other nine were used as controls without plants, simulating the change in land use of native vegetation to agriculture and pasture (Figure 5.2).

Figure 5.2 – Mesocosms experiment in the greenhouse

Since soybean is really sensitive to pH and fertility, all tubes were surface limed with 3 g of $CaCO_3$, aiming a base saturation of 50%, with 2 g of $CaSO_4$, to neutralize Al through the profile, and fertilized with 0.4 g of K_2PO_4. All tubes, with or without plants received this fertilization, so the only effect that would be observed among the tubes would be the origin: Forest or Cerrado, and the plants: Brachiaria, soybean or no plant.

Soybean and Brachiaria were sowed in March 2009. Soybean was first harvested in June 2009. At the same time Brachiaria was also harvested, simulating grazing. For both, the aerial part of the plant was removed from the tubes and its dry mass quantified. In November 2009, soybean was again sowed, simulating a second cultivation. At the same time, Brachiaria was again harvested, simulating a second grazing.

In March 2010 the experiment was finally analyzed. Soil and gas samples were collected for analysis. Plants were harvested again, and immediately after plant removal, GHG measurements were performed, as it will be described below. After GHG analysis, soil was separated and homogenized in two depths, 0-10 cm and 10-30 cm. Homogenization was performed in aseptic plastic bags and all the tools used were previously disinfected with ethanol 80%. Aliquots for DNA, ammonium and nitrate extraction were immediately frozen and kept stored -20 ºC until use. The rest of the soil samples were air dried for use in the chemical analysis.

5.2.3 GHG fluxes

The flows or the emission of CO_2, N_2O and CH_4 were measured in static chambers coupled in PVC pipes at the time of collection (Figure 5.3), adapted from the field chamber method of Steudler et al. (1991). Gas samples were collected with syringes at times 0, 5, 10 and 15 minutes.

Figure 5.3 – Mesocosms at the greenhouse and detail of the lids for GHG survey

Gas samples were analyzed using a GHG GC analyzer. The concentration of CO_2 and CH_4 were determined by a flame ionization detector and N_2O by a Ni electron capture detector (CARVALHO et al., 2010). The fluxes of each gas were determined by calculating the alteration in concentration in the chambers as a function of incubation time. Pressure, chamber size, and temperature were adjusted in the calculation for each sample.

5.2.4 Chemical analysis

Ten g from each sample were ground and sieved to 0.25 mm to determine the total C and N contents, which were analyzed by total combustion (NELSON AND SOMMERS, 1982). Another 10 g were used for pH determination, with an addition of 25 mL of deionized water. After shaking and decanting, pH was measured using an electronic pH meter. Ammonium and nitrate extractions were performed by adding 4 g of frozen soil samples to 40 ml of KCl 1M, agitated for one hour, and filtered in a Whatman quantitative filter no. 40. The extraction was made using KCl 1M and ratio soil extractor 1:10, recommended for tropical soils (VAN RAIJ et al., 2001). The extract was analyzed in a flow injection analysis (FIA) using the conductivity method for ammonium and the nitrate was reduced in a Cd column and NO_2 read by a colorimetrical method (Siqueira et al., 2011).

5.2.5 qPCR analysis

Total DNA was extracted from 0.25 g of soil samples using the Power Soil kit (MoBio, Solana Beach, CA, USA). Extraction was performed according to the manufacturer's instructions, and DNA samples were stored at – 20 ºC.

For each replicate site and day, DNA extraction was performed for three independent soil extractions that were mixed together and used as template for qPCR. Concerns about DNA quality are discussed in Appendices A and B.

Quantitative PCR was performed in an Opticon 2 machine (Bio-Rad) in 96-well plates. All analyses were performed in duplicate. Each reaction was composed of 1 µl of extracted DNA, 0.2 µl of inhibitor resistant enzyme Klentaq (KERMEKCHIEV et al., 2009), 0.5 µg of T4 Gene 32 Protein to increase sensitivity and reduce humic acid inhibition (TEBBE; VAHJEN, 1993) 1 X of KlenTaq buffer, 1 X of EvaGreen, 0.25

mM of dNTP, primers as described (Table 6.1) and molecular grade water to a final volume of 20 µl. Thermo cycler was programmed with an initial denaturation step at 95 ºC 5 min following 40 X 95 ºC 15 s, annealing temperature per 30 s, 68 ºC 30 s, following 5 min 68 ºC, using reading annealing and reading temperature as described (Table 6.1). Melting curve was performed from 72 ºC to 95 ºC. Sloop varied from -3.08 to -3.82 and standard curve r^2 from 0.990 to 0.999.

Standards were made from PCR amplified genes of isolates or soil DNA with each primer set. PCR products were cloned and 3-5 clones sequenced to provide specificity. We preferred to use diluted amplicons as standards instead of plasmids because plasmids can result in distortions in quantification (HOU et al, 2010). Plasmid standards were only used for the quantification of *mcr*A and *pmo*A genes.

Table 5.1 – Primers and amplification conditions for the different genes

Gene	Primer	Annealing (ºC)	Reading (ºC)	Reference
16S rRNA Bac	F563/BSR926	55	80	Claesson et al., 2010
*nor*B	F/R	TD 65-62	83	Dandie et al., 2007
*mcr*A	F/R	55	83	Steinberger et al., 2008
*pmo*A	A189f/A682r	63	85	McDonald et al., 2008

5.2.6 Statistical analysis

Data was analyzed as a completely randomized design. ANOVA analysis was performed and when a significant effect was observed, data was submitted to a Duncan average comparison test ($p<0.05$). A Pearson correlation analysis was performed to test correlation between the analyzed attributes.

5.3 Results

5.3.1 Chemical analysis

Differences among the soil chemical parameters of the samples collected from Forest and Cerrado and also among the different treatments were detected (Table 5.2).

Cerrado areas presented higher C and N content, which could be a consequence of our sampling approach (since we could not sample undisturbed samples from the Cerrado sites, we mixed the soil in the sample and put in the PVC tubes, some litter could be mixed together).

Table 5.2 – Soil chemical parameters (n=3, each n is an average of 3 pseudo replicates)

Sample	pH	C	N	NH_4	NO_3
	H_2O	----- (%) -----		--- mg . g ds $^{-1}$ ---	
No Plant (For 0-10)	5.28 a	2.99 b	0.15 ab	0.64 a	1.39 a
Soybean (For 0-10)	5.25 a	2.68 b	0.11 b	0.57 ab	0.27 b
Brachiaria (For 0-10)	5.44 a	2.85 b	0.12 b	0.46 b	0.13 b
No Plant (Ce 0-10)	4.89 b	3.60 a	0.23 a	0.49 b	1.21 a
Soybean (Ce 0-10)	5.12 ab	3.94 a	0.21 a	0.57 ab	0.24 b
Brachiaria (Ce 0-10)	5.06 ab	3.49 a	0.22 a	0.68 a	0.10 b
No Plant (For 10-30)	4.78 b	1.69 c	0.08 b	0.45 b	0.93 ab
Soybean (For 10-30)	4.81 b	1.81 c	0.08 b	0.56 ab	0.18 b
Brachiaria (For 10-30)	4.90 ab	1.74 c	0.08 b	0.57 ab	0.06 b
No Plant (Ce 10-30)	4.62 b	2.02 bc	0.13 b	0.42 b	0.61 ab
Soybean (Ce 10-30)	4.75 b	1.91 c	0.10 b	0.54 ab	0.14 b
Brachiaria (Ce 10-30)	4.86 b	2.09 bc	0.10 b	0.53 ab	0.11 b

* Values with the same letter in the column are not different by Duncan test ($p<0.05$). For, Forest, Ce, Cerrado, 0-10, depth 0-10cm, 10-30, depth 10-30cm.

Soil pH, as it was expected, is lower within the depth of 10-30 cm. No change in pH, C or N was observed due to plants effect. An effect of the plants in ammonium and nitrate concentrations was observed.

5.3.2 Gene abundance

No clear statistical difference was observed for the abundance of the gene 16S rRNA Bacteria, but a trend of higher abundance in the mesocosms with plants was observed. No difference was observed for the gene *pmo*A. The gene *mcr*A presented great variability, since most of the replicates ranged in the detection limit and could not be detected in some samples. In the depth 10-30 cm, mcrA was detected only in the presence of plants. In the surface it was better detected in

absence of plants and in the forest soil with soybean. The gene *nos*Z presented high variability and a trend of higher abundance in the Cerrado soils (Table 5.3).

Due to technical problems, we could not analyze all pseudo-replications individually before thesis submission, which could explain in part the weak statistical differentiation of the results (Table 5.3).

Table 5.3 – Gene abundance in the mesocosms (n>2, each n is an average of 3 pseudo replicates)

sample	**16S Bac**		***pmo*A**		***mcr*A**		***nos*Z**	
No Plant (For 0-10)	2.5E+09	ab	2.5E+08	a	6.4E+06	ns	2.6E+06	de
Soybean (For 0-10)	5.0E+09	ab	4.7E+08	a	4.7E+06	ns	4.3E+06	cd
Brachiaria (For 0-10)	1.0E+09	b	1.5E+08	a	BDL	ns	1.6E+06	e
No Plant (Ce 0-10)	1.7E+09	b	7.9E+08	a	7.8E+06	ns	2.2E+07	a
Soybean (Ce 0-10)	2.5E+09	ab	3.8E+08	a	BDL	ns	7.9E+06	b
Brachiaria (Ce 0-10)	8.1E+08	ab	1.8E+08	a	BDL	ns	8.1E+06	b
No Plant (For 10-30)	8.7E+08	b	9.4E+07	a	BDL	ns	1.7E+06	e
Soybean (For 10-30)	7.2E+09	ab	2.6E+08	a	3.1E+06	ns	2.7E+06	cd
Brachiaria (For 10-30)	8.5E+09	a	5.8E+08	a	1.9E+06	ns	3.5E+06	cd
No Plant (Ce 10-30)	7.4E+08	b	1.4E+08	a	BDL	ns	4.4E+06	c
Soybean (Ce 10-30)	2.3E+09	ab	3.4E+08	a	1.7E+06	ns	3.9E+06	cd
Brachiaria (Ce 10-30)	7.6E+09	ab	5.4E+08	a	5.5E+06	ns	7.6E+06	b

* Values with the same letter in the columns are not different by Duncan test ($p<0.05$, ns, no significant. For, Forest, Ce, Cerrado, 0-10, depth 0-10cm, 10-30, depth 10-30cm. BDL, below detection limit.

5.3.3 GHG fluxes

The GHG fluxes were highly variable among replicates, making statistical separation difficult (Table 5.4). The only statistical difference observed was in relation to CO_2 flux, Brachiaria growth in Forest soil was higher than the others.

Table 5.4 – GHG production in the mesocosms (n=3, each n is an average of 3 pseudo replicates)

Sample	CO_2 mg C-CO_2.m^{-2}.h^{-1}		CH_4 mg C-CH_4.m^{-2}.h^{-1}		N_2O mg N-N_2O.m^{-2}.h^{-1}	
No Plant (For 0-30)	45.4	b	2.5	ns	5.4	ns
Soybean (For 0-30)	31.9	b	-2.2	ns	3.2	ns
Brachiaria (For 0-30)	114.8	a	-15.7	ns	1.8	ns
No Plant (Ce 0-30)	40.6	b	4.7	ns	6.5	ns
Soybean (Ce 0-30)	28.3	b	-16.9	ns	3.0	ns
Brachiaria (Ce 0-30)	56.0	b	-10.9	ns	0.4	ns

* Values with the same letter in the column are not different by Duncan test (p<0.05), ns, no significant. For, Forest, Ce, Cerrado, 0-10, depth 0-10cm, 10-30, depth 10-30cm.

5.3.4 Correlations

A few correlations were observed. C was correlated with pH and N. The C:N ratio was correlated with N (negative), methane (negative) and CO_2. Nitrate was correlated with methane. The gene 16S rRNA Bacteria was negatively correlated with N and methane, same trend of the ratio C:N, but they were not correlated with each other. The gene *nos*Z was correlated with C and N, and no correlation observed for the gene *pmo*A (Table 5.4).

Table 5.5 – Pearson correlations between selected variables (n=12; the two soil depths, 0-10 cm and 10-30 cm, were analyzed together)

	C	**N**	**N-NH_4**	**N-NO_3**	**C-CH_4**	**C-CO_2**	**N-N_2O**
pH	0.56 *	NS	NS	NS	NS	NS	NS
C	1	**0.93*** **	NS	NS	NS	NS	NS
N	**0.93*** **	1	NS	NS	NS	NS	NS
C:N	NS	-0.65**	NS	NS	-0.51*	0.53*	NS
N-NH_4	NS	NS	1	NS	NS	NS	NS
N-NO_3	NS	NS	NS	1	0.55*	NS	NS
16S Bac	NS	-0.51*	NS	NS	-0.55*	NS	NS
***nos*Z**	0.57**	0.72***	NS	NS	NS	NS	NS
***pmo*A**	NS	NS	NS	NS	NS	NS	NS

* p<0.1, ** p<0.05, *** p<0.01; NS, No Significant. The genes *mcr*A and *nor*B were excluded of the analysis, since most of the replicates were bellow the detection limit.

5.4 Discussion

5.4.1 Chemical analysis

The higher amount of C in Cerrado areas could be consequence of our sampling approach (since we could not sample undisturbed sample from the Cerrado sites, we mixed the soil in the sample and put it in the PVC tubes, and some litter could be mixed together)

Nitrate was generally lower in the mesocosms with a plant, which can be an indicative of plant uptake. In lower pH, soybean has preference per uptake of nitrate instead of ammonium (HAWKINS; ROBBINS, 2010).

General differences in the mesocosms are more related to the origin of the soil than plant effect.

5.4.2 Gene abundance

A trend of lower Bacteria abundance in the mesocosms was observed, specially in the 10-30 cm depth. It can be related to the production of root exudates that stimulate microbial growth (BREMER et al., 2007).

No significant difference was observed for the gene *pmo*A, and mcrA was bellow detection limit for most of the samples. A trend indicate a higher abundance of *mcr*A in the 10-30 cm layer in the treatments with plants, and this can be also related with the root exudates. In the superficial layer, *mcr*A presence was related to absence of plants. It would be related to disturb caused per root growth near the surface. The roots could increase channels near the surface, stimulating the air exchange, inhibiting the growth of the anaerobic metanogenic bacteria. Since liming and fertilizers were applied in surface, it would be expected a higher root activity in the 0-10 cm layer.

For the gene *nos*Z, the abundance was really variable among the mesocosms. The main trend is a higher abundance of the denitrifier bacteria in the surface soil of Cerrado samples. It can be associated with the higher C and N content, substrate for these bacteria, as observed in the correlation analysis (Table 5.5). It could be the consequence of the litter mixed in this layer.

5.4.3 GHG fluxes

A high variability in the gases measurements was observed. The forest soil with Brachiaria presented the higher carbon dioxide flux. No correlation was found with bacteria abundance. It would be associate with a higher fungi presence, or even with root respiration (CARVALHO, 2010). The plants were cut before the measurements, but the roots could be still active during the gas survey.

No significant difference was observed for methane or nitrous oxide. But a trend of production in the mesocosms without plants and consumption in the mesocosms with plants was observed. The *pmo*A gene abundance was the same for all thee mesocosms, but maybe plant can have some effect in the methanotrophic bacteria activity.

5.4.4 Correlations

A few correlations were found. Methane production was correlate with a lower C:N ratio. Usually a lower ratio, means easily decomposable material, and so, more substrate to the methanotrophic bacteria. Also it was correlated with a higher nitrate availability, but with a negative abundance of bacteria. Bacteria can compete for substrate, so the negative correlation would be observed.

The gene *nos*Z was correlated with C and N, as discussed before, indicating a higher presence of the denitrifier bacteria in the Cerrado soils.

5.5 Conclusions

A higher abundance of Bacteria in mesocosms with plants compared to control mesocosms without plants was observed. The gene *nos*Z was correlated with C and N, and consequently, with a higher presence of denitrifier bacteria in Cerrado soils.

Due to technical problems, we could not analyze all pseudo-replications individually at this time.

It is highly likely that the individual analysis of pseudo-replicates, the analysis of additional genes and the use of diversity tools, such as sequencing, will provide better correlations and insights in the influence of these plants in the soils.

6. INFLUENCE OF *G. max* AND *B. brizantha* ON AGRICULTURAL SOILS

The aim of this chapter was to address the effect of plants on the microbial community of agricultural soils of Southwestern Amazon. Mesocosms were built with soil from a newly established agricultural area two years after deforestation. To separate the plant from the land use change effect, control mesocosms without plants were compared to mesocosms with soybeans or Brachiaria. Plant free forest soil represented an additional control. Soil parameters such as pH, C, N, and gas fluxes of carbon dioxide, methane, and nitrous oxide were determined and correlated with microbial gene abundance. Agricultural soils differed significantly from Forest soils. Forest soils presented higher microbial biomass and consequently gene, and microbial, abundance. The absence of plants yielded more nitrogen accumulation mostly in the form of nitrate. A correlation was found between available nitrate and denitrifier bacteria abundance. Also, a correlation was found between available nitrate and nitrous oxide emissions, suggesting that plants can compete for available nitrogen, reducing nitrous oxide emissions.

Key words: Mesocosms. Rhizosphere. New agricultural area.

6.1 Introduction

In chapter 5, with aim to indentify plant effect on the soil samples, a mesocosms experiment was set up. For that experiment, soil from native Forest and Cerrado was used, simulating land use change from native vegetation to agriculture and pasture.

However, land use change involves more changes than liming, fertilization and change in the plant coverage. It involves deforestation, burn, root removal, and till operations, such as harrowing and leveling of the soil.

To detect more realistic differences, a new experiment was set up, surveying soil of an area that has been cleaned from Forest to Agriculture, in the Jaguaruna Farm, as described in Chapter 2.

The aim of this chapter was to study the effect of Brachiaria and soybean in the abundance of soil microbial groups related to the C and N cycles and to correlate this to soil parameters, such pH, C, N, and to the flux of the gases carbon dioxide, methane, and nitrous oxide.

6.2 Material and Methods

6.2.1 Survey

Soil was surveyed in agriculture and forest areas of Jaguaruna Farm, Porto dos Gauchos, MT (Chapter 2). Soil was dug from the three replicate sites. First, individual samples were taken from the five pseudo-replicate plots and mixed together, making one mixed sample per replicate site. In total, six soil samples were surveyed: three samples from the replicate sites of agriculture soils and three samples from the replicate sites of Forest soils.

These soil samples were gently surveyed by colleagues of the Molecular Biology Laboratory at CENA-USP, who were surveying soils in the region. Unfortunately, the amount of the soil surveyed was not enough to fill big tubes, as the experiment before. Thus, instead of using tubes of 30 x 15 cm as in Chapter 5, the soil was transferred to tubes of 20 x 10 cm.

The samples of the agricultural soils were transported in a pickup truck, but only a replicate soil of Forest was surveyed. A week later, a colleague living in Sinop surveyed soil samples from all three replicate sites in Forest soils and sent the samples by airplane to Piracicaba. Thus, the Forest soils were about two weeks fresher than the agricultural soils when the experiment was set up in the greenhouse.

6.2.2 Experiment in greenhouse

The experiment was conducted in a greenhouse at CENA-USP. The surveyed soil was divided in 36 tubes, with 800 g of dry soil added to each tube. Groups of nine tubes were separated according to each agricultural replicate site, and groups of three tubes for each Forest replicate sites. Each individual tube was considered a mesocosms.

PVC tubes of 20 x 10 cm were used, and sealed at the bottom with a plastic cap. A hole was made in the center to drain excess water in the tubes. An autoclaved felt square was put over the hole to prevent losing soil. All the tubes were disinfested with 80% ethanol prior to adding soil inside them.

For the agricultural soils, Brachiaria was sown in three tubes, soybean in three tubes, and three tubes were kept as controls without plants. Since soil was surveyed from three replicate sites, a total of 27 mesocosms for the agriculture soils were made. Three tubes per replicate site were kept as no plant controls, totaling nine tubes for the Forest soils (Figure 6.1).

Brachiaria and soybean were sowed in November 2009, simulating cultivation in the agriculture soils. No liming or fertilizers were added, since the agricultural soil was already prepared for soybean cultivation. Every two days, each mesocosm was watered with deionized water. All the water used was pre-treated by ultraviolet light, in order to avoid microbial contamination (standard system of CENA-USP greenhouse).

In March 2010, the experiment was analyzed. Soil and gas samples were collected for analysis. Plants were harvested, and immediately after plant removal, GHG measurements were performed, as it will be described below. After GHG analysis, soil was homogenized in aseptic plastic bags. All the tools used were

previously disinfected with 80% ethanol. Aliquots of soil for DNA, ammonium and nitrate extraction were immediately frozen and stored at -20 ºC until use. The rest of the soil samples were air-dried for use in the chemical analysis.

Figure 6.1 – Mesocosms in the greenhouse

6.2.3 GHG analysis

The fluxes or the emission of CO_2, N_2O and CH_4 were measured in static chambers coupled in PVC pipes at the time of collection, adapted from Steudler et al. (1991). Gas samples were collected with syringes on times 0, 5, 10 and 15 minutes.

Gas samples were analyzed using a GHG GC analyzer. The concentration of CO_2 and CH_4 were determined by a flame ionization detector and N_2O by a Ni electron capture detector (CARVALHO et al., 2010). The fluxes of each gas were determined by calculating the alteration in concentration in the chambers as a function of incubation time. Pressure, chamber size, and temperature were adjusted in the calculation for each sample.

6.2.4 Soil analysis

Ten g from each sample were ground and sieved to 0.25 mm to determine the total C and N contents, which were analyzed by total combustion (NELSON; SOMMERS, 1982). Another 10 g were used for pH determination, with an addition of 25 mL of deionized water. After shaking and decanting, pH was measured using an electronic pH meter. Ammonium and nitrate extractions were performed by adding 4 g of frozen soil samples to 40 ml of KCl 1M, agitated during one hour, and filtered in a Whatman quantitative filter nº. 40. The extraction was made using KCl 1M and ratio soil extractor 1:10, recommended for tropical soils (VAN RAIJ et al., 2001). The extract was analyzed in a FIA using the conductivity method for ammonium and the nitrate was reduced in a Cd column and NO_2 read by a colorimetrical method (SIQUEIRA et al., 2011).

The soil microbial biomass carbon (MBC) was determined by using the fumigation-extraction method (VANCE et al., 1987). For this analysis, fumigated and non-fumigated aliquots of 25 g of soil samples were extracted with 0.5 M K_2SO_4. Organic C in the sulfate extracts was oxidized by acid dichromate digestion. The excess dichromate was determined by back-titration with $(NH4)_2Fe(SO4)_2$. MBC was calculated from the difference between organic C content in fumigated and non-fumigated soils using kEC of 0.33 (FEIGL et al., 1995).

6.2.5 qPCR analysis

Total DNA was extracted from 0.25 g of soil samples using the Power Soil kit (MoBio, Solana Beach, CA, USA). Extraction was performed according to the manufacturer's instructions, and DNA samples were stored at – 20 ºC.

For each replicate site and day, DNA extraction was performed for three independent soil extractions that were mixed together and used as template for qPCR. Concerns about DNA quality are discussed in Appendices A and B.

Quantitative PCR was performed in an Opticon 2 machine (Bio-Rad) in 96-well plates. All analyses were performed in duplicate. Each reaction was composed of 1 µl of extracted DNA, 0.2 µl of inhibitor resistant enzyme Klentaq (KERMEKCHIEV et al., 2009), 0.5 µg of T4 Gene 32 Protein to increase sensitivity and reduce humic acid inhibition (TEBBE; VAHJEN, 1993) 1 X of KlenTaq buffer, 1 X of EvaGreen, 0.25

mM of dNTP, primers as described (Table 6.1) and molecular grade water to a final volume of 20 µl. Thermo cycler was programmed with an initial denaturation step at 95 ºC 5 min following 40 X 95 ºC 15 s, annealing temperature per 30 s, 68 ºC 30 s, following 5 min 68 ºC, using reading annealing and reading temperature as described (Table 6.1). Melting curve was performed from 72 ºC to 95 ºC. Sloop varied from -3.08 to -3.82 and standard curve r^2 from 0.990 to 0.999.

Standards were made from PCR amplified genes of isolates or soil DNA with each primer set. PCR products were cloned and 3-5 clones sequenced to provide specificity. We preferred to use diluted amplicons as standards instead of plasmids because plasmids can result in distortions in quantification (HOU et al, 2010). Plasmid standards were only used for the quantification of *mcr*A and *pmo*A genes.

Table 6.1 – Primers and amplification conditions for the different genes

Gene	Primer	Anealing (ºC)	Reading (ºC)	Reference
16S rRNA Bac	F563/BSR926	55	80	Claesson et al., 2010
*nor*B	F/R	TD 65-62	83	Dandie et al., 2007
*mcr*A	F/R	55	83	Steinberger et al., 2008
*pmo*A	A189f/A682r	63	85	McDonald et al., 2008

6.2.6 Statistical analysis

Data was analyzed as a completely randomized design. ANOVA analysis was performed and when significant effect was observed, data was submitted to a Duncan average comparison test ($p<0.05$). A Pearson correlation analysis was performed to test the correlation between the analyzed attributes.

6.3 Results

6.3.1 Soil analysis

Differences were observed between the forest soil and the agriculture soil. MBC and nitrate were higher in the Forest soil. Ammonium was higher in the Forest soil than the soybean mesocosms. No differences were observed for C, N, and C:N, but pH was lower in the Forest soil than in the other mesocosms (Table 6.2).

Table 6.2 – Chemical analysis of the mesocosms' soils (n=3, each n is an average of three pseudo replicates)

Mesocosms	pH H_2O	C %	N %	C:N	NH_4 --------	NO_3 µg . g ds $^{-1}$	MBC --------
Soybean	5.3 a	2.7 a	0.17 a	16 a	0.28 b	0.24 c	22 b
Brachiaria	5.4 a	2.8 a	0.16 a	17 a	0.35 ab	0.14 c	42 b
No Plant (Ag)	5.2 a	2.8 a	0.17 a	16 a	0.30 ab	1.14 b	36 b
No Plant (Fo)	3.5 b	2.9 a	0.18 a	16 a	0.38 a	1.84 a	136 a

*Values with the same letter in the line are not different by Duncan test ($p<0.05$). Ag, Agriculture soil, Fo, Forest soil.

6.3.2 Gene abundance

Gene abundance was higher in the forest soil for the genes 16S rRNA Bacteria, *nos*Z and *pmo*A. Both, *mcr*A and *nor*B presented problems in amplification; most of the samples were below the detection limit. The *mcr*A results were not reliable and are not shown. The norB result reached close to the detection limit for most replicates and no statistical difference was observed among the mesocosms (Table 6.3).

Table 6.3 – Gene abundance in the mesocosms' soils in copies . g ds^{-1} (n=3, each n is an average of three pseudo replicates).

Mesocosms	16S Bac	*nos*Z	*pmo*A	*nor*B
Soybean	1.E+07 b	7.E+05 b	2.E+06 b	1.E+06 ns
Brachiaria	1.E+07 b	5.E+05 b	2.E+06 b	2.E+06 ns
No Plant (Ag)	1.E+07 b	3.E+05 b	3.E+06 b	BDL ns
No Plant (Fo)	6.E+07 a	2.E+06 a	7.E+06 a	2.E+06 ns

*Values with the same letter in the line are not different by Duncan test ($p<0.05$); ns, no significant. Ag, Agriculture soil, Fo, Forest soil.

6.3.3 GHG fluxes

The greenhouse gas fluxes in the mesocosms varied greatly among replicates, resulting in no significant differences for most of the gases and treatments. Carbon dioxide was higher for the agricultural soils (Table 6.4).

Table 6.4 – GHG flux of the mesocosms (n=3, each n is an average of three pseudo replicates)

Mesocosms	C-CO_2 mg $m^{-2}.h^{-1}$	C-CH_4 mg $m^{-2}.h^{-1}$	N-N_2O µg $m^{-2}.h^{-1}$
Soybean	133.7 a	-9.8 ns	7.2 ns
Brachiaria	149.4 a	-1.0 ns	9.9 ns
No Plant (Ag)	166.1 a	1.9 ns	27.8 ns
No Plant (Fo)	21.7 b	-8.3 ns	31.2 ns

*Values with the same letter in the line are not different by Duncan test ($p<0.05$); ns, no significant. Ag, Agriculture soil, Fo, Forest soil.

6.3.4 Correlations

Some correlations were observed among the variables. There was a contrast between pH and MBC of agricultural soil and Forest soil, and several correlations were observed for these two parameters. There was a correlation between eac, with ammonium, nitrate, and CO_2. MBC presented strong correlations with the genes16S rRNA Bacteria and *nos*Z. The gas, CH_4, presented a correlation with the C:N ratio; N_2O presented a correlation with nitrate and the gene pmoA; and the gene pmoA presented a correlation also with nitrate (Table 6.4).

Table 6.4 – Pearson correlations between selected variables (n=12)

Attribute	MBC	$N\text{-}NH_4$	$N\text{-}NO_3$	$C\text{-}CH_4$	$C\text{-}CO_2$	$N\text{-}N_2O$
pH	**-0.93 *****	-0.58 **	**-0.74 *****	NS	**0.70 ****	NS
C	NS	NS	NS	NS	NS	NS
N	NS	NS	NS	NS	NS	NS
C:N	NS	NS	NS	0.50*	**0.74 *****	NS
MBC	1	0.64 **	**0.78 *****	NS	**-0.74 ****	NS
$N\text{-}NH_4$	0.64 **	1	NS	NS	NS	NS
$N\text{-}NO_3$	0.54 *	NS	1	NS	-0.50 *	0.52 *
16S Bac	**0.71 ****	NS	0.54 *	NS	**0.71 ****	NS
nosZ	**0.83 *****	NS	**0.68 ****	NS	-0.69 **	NS
pmoA	0.65 **	NS	**0.92 *****	NS	-0.56 *	0.51*

* p<0.1, ** p<0.05, *** p<0.01; NS, No Significant. The genes *mcr*A and *nor*B were excluded of the analysis, since most of the replicates were bellow the detection limit.

6.4 Discussion

6.4.1 Soil analysis

As expected, pH of the soil from the Forest was lower than the agricultural soil. The agricultural soil received lime. Again, no differences in pH, C and N were observed due to plant effect, but differences in ammonium and nitrate (Chapter 5). Nitrate was also used by the plants in this experiment an variable effect in ammonium observed.

Nitrate, ammonium and MBC were higher in the Forest soils. The agricultural soil suffered great impact due to till practices and would be expected a lower MBC than Forest (FRAZÃO et al., 2010).

6.4.2 Gene abundance

A higher abundance of genes was observed in the Forest soils, according to the higher biomass result. Two factors would be related to this. First is the impact of the till practices to the soil (FRAZÃO et al., 2010). Revolving the soil expose the bacteria to the environment and to the sun, what can in a first moment cause suppression in the microbial activity. Second the soil survey. Samples were firs

surveyed in the agriculture sites and transported by pickup truck. The soil was mixed before transportation and it could have caused some impact to the microbial community. For the other experiment soil was transported in soil cores, which could have protected the microbiota. The Forest soil samples were sent two week later by airplane, and they could have suffered less impact.

No differences were observed among the genes and the agricultural soils with or without plants. The only exception was the gene *nor*B, that was not detected in absence of plant. Plant exudates can stimulate denitrifiers communities (BREMER et al., 2007).

6.4.3 GHG fluxes

The only significant effect was in relation to carbon dioxide that was lower in the Forest soils. It was a quite a surprise, since it presented a higher MBC, and consequently gene abundance, it would be expected a higher activity in this soils (FRAZÃO et al., 2010).

The soils without plant presented a trend of higher nitrous oxide flux, what could be associate with the higher nitrogen available. No significant difference was observed for the nitrous oxide emission, but the correlation analysis showed a significant correlation between nitrate and nitrous oxide, supporting this trend.

6.4.4 Correlations

Several correlations between pH and analyzed parameters were observed. It can be related that Forest and agriculture soil presented several differences and also distinct pH. Also, several attributes were related with MBC, especially gene abundance. However, MBC was approximately 5 times higher in the Forest soils than the others, and so correlation with a higher abundance of genes, and consequently microbes, would be expected.

A correlation between nitrate and nitrous oxide was observed, as a correlation between nitrate and *nos*Z; and, between nitrate and bacteria. It supports the trends observed for the soils without plants. A higher available nitrogen made the denitrification process favorable.

6.5 Conclusions

Differences between agriculture and Forest soils were observed. Forest soils presented higher microbial biomass and consequently gene, and microbial, abundance. The absence of plants yielded more nitrogen accumulation mostly in the form of nitrate. A correlation was found between available nitrate and denitrifier bacteria abundance. Also, a correlation was found between available nitrate and nitrous oxide emissions, suggesting that plants can compete for available nitrogen, reducing nitrous oxide emissions.

7. CORRELATION BETWEEN MICROBIAL GENE ABUNDANCE AND THE RESPECTIVE C, N, AND GHG MEASUREMENTS IN A FIELD SURVEY

Abstract

Analyses *in situ* provide better insights about how the biogeochemical processes occur naturally. In this chapter we discuss changes in soil parameters at the beginning of the rainy season, before soybean seedlings are set, and in the month of January during higher activity (i.e. high soil hydro balance, high temperature, and soybean growth, immediately after flowering). The objective of this chapter was to correlate soil chemical attributes, greenhouse gases (CO_2, CH_4 and N_2O) and the abundance of processes-related microbial genes (*amo*A, *nir*K, *nir*S, *nor*B, *nos*Z, *mcr*A and *pmo*A) surveyed in soil samples from the Amazon Forest and land that underwent conversion to pasture and agriculture. First we show how land use changes the chemical attributes of soil such as higher pH in soybean fields and pastures, the liming effect, and higher nutrient levels in soybean soils, the fertilizer effect. During the survey period, the forest presented higher microbial activity, resulting in higher nitrate availability and N_2O emission, which correlated with higher abundance of processes-related genes. The concentrations of nitrate and N_2O emissions were lower in agriculture and pasture samples. CO_2 emission was higher in soybean fields in their 2^{nd} year of cultivation after deforestation. Forest and soybean fields during the 2^{nd} year served as a sink for CH_4, while pasture served as a source. Our results validated the use of quantitative PCR to provide a better understanding of the processes, but a larger temporal and spatial analysis is needed to infer statements about more comprehensive process dynamics as a result of land use change. The genes *nir*K, *nos*Z, and 16S rRNA presented the best correlations with soil processes and we discuss needs for improvement of these techniques.

Key words: GHG. Land use change. Gene abundance.

7.1 Introduction

Several studies have been done in Southwestern Amazon to evaluate the impact of land use change on the soil carbon and green house gases emission (NEIL et al., 2007; CARVALHO et al., 2010). A general trend is that land use change from native vegetation to pasture or agriculture creates a lost of carbon in the first years of the conversion, followed by an accumulation of carbon if conservational managements are chosen, such as agricultural no-tillage systems, or carbon lost when the soil receives inadequate management, such as excessive tillage or pasture degradation (CARVALHO, 2010; FRAZÃO et al., 2011).

Thus, it is expected that high microbial activity in the first years will occur after deforestation due to decomposition of roots of the forest and tillage. After pasture and agriculture establishment, especially in no-tillage systems, it is expected that CO2 rates are similar between native vegetation, pasture and agriculture lands. Usually it is expected that higher methane emission in pasture sites will occur due to the animals' impact in compacting the soil and adding feces and urine to the soil; and higher N_2O emission in the agriculture soils due to application of nitrogen fertilizers or to decomposition of the residues of the crops, especially N-fixing symbiotic plants, as soybean (TORRES et al., 2005).

Some knowledge is already available about the carbon and greenhouse gas dynamic, but little is known about the microbes related to these processes, on the C and N cycles. Several papers has shown the potential of using qPCR targeting functional genes, such as *nos*Z, to monitoring this parameters in soil (HENRY et al., 2006; DANDIE et al., 2007; MORALES et al., 2010) but it was never used in Southwestern Amazonia soils.

In this chapter we analyze *in situ* how microbial functional groups shifts due to land use change by studying a native forest, a pasture site of 25 years, an agricultural site of 25 years and a new agricultural site in its third year after deforestation. We hypothesize a higher microbial abundance in the forest due to higher litter and matter cycling, and also in the new agricultural site, due to the trends described above.

One characteristic of Southwestern Amazonia is a dry period between May and September. So, we hypothesized that land use effect, sum of plant coverage, human interference and soil characteristics, is lower at the end of the dry period because all the sites are suffering hydric deficit and that the agricultural site is in fallow, resulting in lower microbial activity.

Thus, mostly of our survey and analytical efforts were performed in January, since it was expected that higher microbial activity, due to a higher soil hydro balance, higher temperature average, and soybean is at maximum growth, immediately after flowering.

7.2 Material and Methods

7.2.1 Survey

This study was conducted in Farm Luiza, Farm São José and Farm Jaguaruna, as previously described (Chapter 2). Soil samples were surveyed during two different periods, in November 2010, at the start of the rain period, and prior to soybean seedling and January, in middle of the rain period, and immediately after soybean flowering (stage R3 in the survey moment).

In January, soil survey was performed together with gas survey, with the aim of correlating microbial parameters with GHG quantification. Surveys were performed on January 15, 17 and 19 at the Farm Luiza and São José (Forest, Pasture ~25 years and Soybean 25 years) and on January 16 at Fazenda Jaguaruna (Forest and Soybean second year).

In the November 2010 survey, soil cores were collected in each of the three replicated sites, as described before (Chapter 2). In the January surveys, soil cores were collected immediately after GHG sampling and inside the chamber base to ensure that the soil core corresponds to its respective gas sample. GHG sampling will be described below. All the samples were processed with caution to avoid contamination and all plastic tubes used to extract the soil cores were previously disinfested with ethanol 80%.

Immediately after sampling, the soil cores were preserved on ice, and frozen at – 20 ºC on the same day of the survey. Soil was preserved at this temperature for one week, until it was used to extract DNA, nitrate and ammonium.

7.2.2 Chemical analysis

Soil samples were processed as described by CARVALHO et al. (2007). First, samples were air-dried and sieved at 2 mm. Soil pH was measured in 0.01 M $CaCl_2$. Exchangeable cations ($Ca^{2+,}$ and Mg^{2+}) and available P were extracted by using ion exchange resins and trace elements by DTPA-TEA, according to Raij and Quaggio (1983). From each sample 10 g of soil were ground and sieved using 0.25 mm for determination of Total Organic Carbon (C) and Total Organic Nitrogen (N) which were determined using a Carbon Nitrogen Analyzer-LECO CN-2000 (CARVALHO et al., 2010).

7.2.3 qPCR analysis

Total DNA was extracted from 0.25 g of soil samples using the Power Lyzer kit (MoBio, Solana Beach, CA, USA). Extraction was performed according to the manufacturer's instructions, and DNA samples were stored at – 20 ºC.

For each replicate site and day, DNA extraction was performed for five independent soil extractions that were mixed together and used as template for qPCR.

Quantitative PCR was performed in an Opticon 2 machine (Bio-Rad) in 96-well plates. All analyses were performed in duplicate. Each reaction was composed of 1 µl of extracted DNA, 0.2 µl of inhibitor resistant enzyme Klentaq (KERMEKCHIEV et al., 2009), 0.5 ug of T4 Gene 32 Protein to increase sensitivity and reduce humic acid inhibition (TEBBE; VAHJEN, 1993) 1X of KlenTaq buffer, 1 X of EvaGreen, 0.25 mM of dNTP, primers as described (Table 6.1) and molecular grade water to a final volume of 20 µl. Thermo cycler was programmed with an initial denaturation step at 95 C 5 min following 40 X 95 ºC 15 s, annealing T per 30 s, 68 ºC 30 s, following 5 min 68 ºC, using reading annealing and reading temperature as described (Table 7.1). Melting curve was performed from 72 ºC to 95 ºC. Sloop varied from -3.08 to -3.82 and standard curve r^2 from 0.990 to 0.999.

Standards were made from PCR amplified genes of isolates or environmental DNA with each primer set. PCR products were cloned and 3-5 clones sequenced to provide specificity. We preferred to use diluted amplicons as standards instead of plasmids because plasmids can result in distortions in the quantification. Plasmid standards were only used for the quantification of *mcr*A and *pmo*A genes.

Table 7.1 – Primers and amplification conditions for the different genes

Gene	Primer	Anealing (ºC)	Reading (ºC)	Reference
16S rRNA Bacteria	F563/BSR926	55	80	Claesson et al., 2010
16S rRNA Archaea	915F/1059R	61	82	Yu et al., 2005
18S rRNA Fungi	EF4/Fung 5r	59	80	Elsas et al., 2000
*amo*A	F/R	63	82	Islam et al., 2009
*nir*K	F1aCu/R3Cu	TD 65-60	83	Hallin et al., 1999
*nir*S	Cd3aF/R3cd	TD 65-60	83	Throbäck et al., 2004
*nor*B	*cno*rBF/R	TD 65-62	83	Dandie et al., 2007
*nos*Z	2F/2R	TD 65-62	83	Henry et al., 2006
*mcr*A	Mlas/*mcr*AR	55	83	Steinberger et al., 2008
*pmo*A	A189f/A682r	63	85	McDonald et al., 2008

7.2.4 GHG analysis

The chamber method of the Environmental Biogeochemical Laboratory at CENA/USP was used for the analysis of GHG, as described by Carvalho et al. (2010), with small modifications. Gas emission measurements were made using a two-piece static polyvinyl chloride plastic chamber (STEUDLER et al., 1989).

Five chamber-bases separated from each other from approximately 20 meters were inserted approximately 2 cm into the soil at each study site. Initial headspace gas samples were collected using 20-ml nylon syringes at the beginning of the incubation and at 10 and 20 min thereafter. Syringes were flushed three times with chamber air to mix the chamber headspace atmosphere and after 10 ml of air samples was surveyed and transferred to a glass tube hermetically closed for storage until analysis at CENA/USP. All samples were analyzed within 20 days after

the survey. Chamber air and soil temperatures at 5 and 10 cm depths were measured for one representative chamber for all sites. Barometric pressure was measured at the beginning of each incubation period (Figure 7.1).

Figure 7.1 – GHG survey at the Forest site (A), Soybean site (B) and detail of the base and chamber at the Pasture site (C and D)

Gas samples were analyzed using a GHG GC analyzer. The concentration of CO_2 and CH_4 were determined by a flame ionization detector and N_2O by a Ni electron capture detector (CARVALHO et al., 2010). The fluxes of each gas were determined by calculating the alteration in concentration in the chambers as a function of incubation time. Pressure, chamber size, and temperature were adjusted in the calculation for each sample.

7.2.5 Statistical analysis

The sampling design was completely randomized, with three replicate sites for each land use studied and five pseudo-replicates per site. The area was located in similar topography, edaphic and climatic conditions, assuming the random design. ANOVA analysis was performed and when significant effect was observed, Duncan average comparison was performed ($p<0.1$, since biological measurements result in great variability within the replicates). A Pearson correlation analysis was performed to test correlation between the analyzed attributes.

7.3 Results

Chemical results were presented only once, and corresponded to the survey of January 2011. For the determination of GHG and gene abundance, our sampling effort was concentrated on January 2011, but a previous survey of gene abundance was performed prior to the soybean seedling in the beginning of the rain season on November 2010.

Based on the data, we concluded that the trends presented in the average table and graphs of the samples were the same as the complete data set. Therefore we prefer to present the simplest data visual option for ease of interpretation. The complete data set is presented in Appendix C.

7.3.1 Environmental and soil chemical attributes

There was no significant difference among the sites in relation to water content (WC) and Air Temperature (Air Temp). A difference was observed for mineral nitrogen and soil temperature (Table 7.2 and Figure 7.3).

Table 7.2 – Environmental attributes in the surveyed areas (Duncan $p< 0.05$)

Attribute	Units	Forest		Soy 2 y		Soy 25 y		Pasture	
Air Temp	ºC	26.4	a	29.2	a	28.8	a	28.3	a
Soil Temp 5 cm	ºC	22.0	b	24.5	a	22.0	b	23.3	ab
Soil Temp 10 cm	ºC	21.4	b	23.5	a	22.0	ab	23.7	a
Soil WC	%	23.1	a	23.3	a	23.2	a	23.1	a
Soil NH_4-N	mg.kg ds^{-1}	3.8	a	0.7	b	2.2	b	2.6	b
Soil NO_3-N	mg.kg ds^{-1}	2.7	a	0.8	b	0.7	b	0.9	b

* Full data set per plots is presented on Appendix C. Values with the same letter in the line are not different by Duncan test ($p<0.05$).

Some chemical differences among land uses were evident, such as pH, nutrient levels, C, N, CEC and some trace elements (Table 7.3).

Table 7.3 – Soil chemical attributes in the surveyed areas (Duncan p<0.05)

Attribute	Unit	Forest	Soybean 2 y	Soybean 25 y	Pasture
pH	($CaCl_2$)	3.9 c	5.1 a	5.0 a	4.6 b
C	$g.kg^{-1}$	2.95 a	2.63 b	2.25 d	2.47 c
N	$g.kg^{-1}$	0.18 a	0.14 b	0.14 b	0.15 b
OM *	$g.kg^{-1}$	44.7 a	39 b	35.7 b	38 b
CEC	$cmol_c.dm^{-3}$	91 a	80 b	68 c	69 c
K	$cmol_c.dm^{-3}$	0.8 b	0.9 b	1.3 a	0.8 b
Ca	$cmol_c.dm^{-3}$	2.0 c	25.0 a	27.7 a	11.7 b
Mg	$cmol_c.dm^{-3}$	2.0 c	19.7 a	6.3 b	5.7 b
P	$mg.dm^{-3}$	4.3 c	16 a	13.7 a	3.3 c
S	$mg.dm^{-3}$	9.0 b	23.3 a	13.3 b	9.7 b
B	$mg.dm^{-3}$	0.35 a	0.28 c	0.26 bc	0.23 b
Cu	$mg.dm^{-3}$	0.2 b	0.7 a	0.4 b	0.1 b
Fe	$mg.dm^{-3}$	273 a	94 b	59 c	118 b
Mn	$mg.dm^{-3}$	2.6 a	1.2 b	0.9 b	1 b
Zn	$mg.dm^{-3}$	0.3 b	2.2 a	1.9 a	0.4 b
Sand	$g.kg^{-1}$	405 a	386 ab	387 b	357 b
Silt	$g.kg^{-1}$	94 a	80 a	86 a	67 b
Clay	g.kg-1	501 a	534 a	527 a	576 b

* OM, Organic Matter. *Values with the same letter in the line are not different by Duncan test (p<0.05).

7.3.2 Gene abundance of the process-related genes

The first characterization of gene abundance in the soils was made on November 2010, in the beginning of the rain season. No statistical significance was observed among land uses (Duncan p<0.05), corroborating with the hypothesis that land use effect on the microbial community in the dry season is low (Figure 7.2).

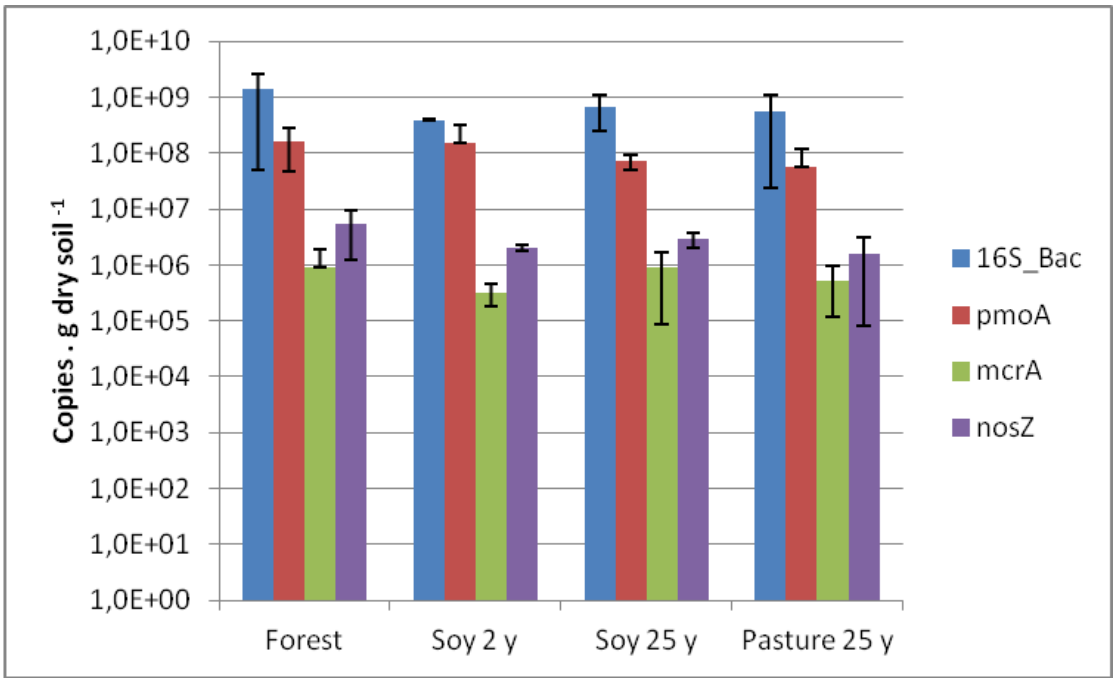

Figure 7.2 – Gene abundance in the beggining of the rain season (Nov. 2010)

Since we did not detect correlation among genes, processes and days (Table 7.4), we refer to the results of the second survey performed on January 2011 as average per land use (Table 7.4, Figure 7.3). The full data set separated by days is available in Appendix C. It is possible to see variation in the data caused by different land uses compared to what was observed in November. This means that the dry season and fallow had an impact in the microbial community.

Table 7.4 – Gene abundance in the different land uses, in copies . g . d.s.$^{-1}$ (Duncan p<0.1)

Gene	Forest		Soy 2 y		Soy 25 y		Pasture	
16S Bac	2.8E+09	a	2.1E+08	b	3.5E+08	b	1.1E+09	ab
16S Arc	4.2E+06	b	7.4E+06	ab	1.3E+07	a	1.0E+07	ab
18S Fung	5.0E+06	a	1.6E+05	b	2.7E+05	b	6.1E+06	a
*amo*A	3.3E+05	c	1.5E+06	c	7.5E+06	a	4.9E+06	b
*nor*B	2.2E+06	a	5.7E+05	a	2.6E+06	a	1.2E+06	a
*nir*K	1.5E+06	b	5.9E+05	b	3.4E+06	a	4.3E+06	a
*nir*S	2.1E+06	ab	4.5E+05	b	8.5E+05	b	4.9E+06	a
*nos*Z	8.0E+06	a	1.7E+06	b	2.2E+06	b	8.3E+06	a
*mcr*A	2.0E+05	a	1.4E+04	c	2.3E+04	c	1.2E+05	a
*pmo*A	1.9E+07	a	9.8E+06	a	1.0E+07	a	4.9E+07	a

* Values with the same letter in the column are not different by Duncan test (p<0.1).

The data for *mcr*A and *nor*B varied near the detection limit for these genes, especially *mcr*A for soybean samples (1.10^3 copies. µl of DNA, or 4.10^5 copies. g dry soil^{-1}). Several of the replicates were not detected, causing variation in the values and no statistical difference was observed among the land uses. These genes also did not present good correlation with the environmental parameters measured, which is an indication that improvement in the techniques for qPCR of these genes is need, as later discussed.

Differences in the abundance of the processes-related genes and the total community abundance for that gene were observed (Table 7.5).

Table 7.5 – Gene abundance in the different land uses (Duncan p< 0.1)

Gene relations	Forest		Soy 2 y		Soy 25 y		Pasture	
Arc/Bac	0.2%	b	3.5%	ab	3.7%	a	0.9%	ab
Fung/Bac	0.2%	a	0.1%	a	0.1%	a	0.5%	a
Fung/Arc	121%	a	2%	b	2%	b	61%	ab
norB/Bac	0.1%	a	0.3%	a	0.7%	a	0.1%	a
nirK/Bac	0.1%	b	0.3%	ab	1.0%	ab	0.4%	a
nirS/Bac	0.1%	a	0.2%	a	0.2%	a	0.4%	a
nosZ/Bac	0.3%	a	0.8%	a	0.6%	a	0.7%	a
pmoA/Bac	0.7%	b	4.7%	a	3.0%	ab	4.4%	a
mcrA/Arc	4.8%	a	0.2%	b	0.2%	b	1.2%	a
amoA/Arc	7.9%	b	20.9%	ab	56.4%	a	48.7%	a

* Values with the same letter in the column are not different by Duncan test (p<0.1).

7.3.3 GHG measurements

We observed differences in GHG flux between land uses (Table 7.6, Figure 7.3). The complete data set for the GHG flux by surveyed days is available in Appendix C.

Table 7.6 – GHG flux per land use (Duncan p< 0.1)

GHG	Unit	Forest		Soy 2 y		Soy 25 y		Pasture	
CH_4	mg C-CH_4 $m^{-2}.h^{-1}$	-6.8	b	-13.7	b	0.8	b	45.7	a
CO_2	mg C-CO_2 $m^{-2}.h^{-1}$	128.6	b	258.2	a	97.7	b	110.6	b
N_2O	ug N-N_2O $m^{-2}.h^{-1}$	33.1	a	9.2	b	3.8	b	3.6	b

* Values with the same letter in the column are not different by Duncan test (p<0.1).

7.3.4 Correlations among genes and processes

Data of GHG, chemical and genes abundances presented significant correlations (Table 7.7).

Table 7.7 – Pearson correlation between selected attributes (n=21)

	C	**N**	**N-NH_4**	**N-NO_3**	**C-CH_4**	**C-CO_2**	**N-N_2O**
C	1	**0.91 *****	0.37 *	0.69 ***	-0.38 *	NS	**0.60 *****
OM	0.64 ***	0.64 ***	0.62 ***	**0.76 *****	NS	NS	0.56 ***
$C.N^{-1}$							
N	**0.91 *****	1	0.51 **	**0.75 *****	-0.38 *	NS	**0.64 *****
N-NH_4	0.37 *	0.51 **	1	**0.74 *****	NS	NS	NS
N-NO_3	0.69 ***	**0.75 *****	**0.74 *****	1	NS	NS	0.37 *
S-SO_4	NS	-0.39 *	NS	NS	NS	**0.66 *****	NS
pH	-0.83 ***	-0.89 ***	-0.67 ***	**-0.82 *****	NS	NS	-0.55 **
Cu	NS	NS	NS	NS	NS	0.48 **	NS
16S Bac	0.47 **	0.46 **	NS	0.56 ***	NS	NS	**0.64 *****
16S Arc	-0.37 *	NS	NS	NS	NS	NS	NS
18S Fung	0.36 *	0.41 *	0.44 **	0.55 ***	NS	NS	NS
***amo*A**	**-0.77 *****	-0.68 ***	-0.37 *	-0.61 ***	NS	-0.41*	-0.59 ***
***nir*K**	0.44 **	0.49 **	0.54 **	**0.77 *****	NS	NS	0.42 *
***nir*S**	NS	NS	NS	NS	0.41 *	NS	NS
***nor*B**	NS	NS	NS	NS	NS	NS	NS
***nos*Z**	0.67 ***	0.68 **	0.46 **	**0.79 *****	NS	NS	**0.61 *****
***mcr*A**	0.54 **	0.57 ***	0.41 *	0.64 ***	NS	NS	0.44 **
***pmo*A**	0.60 ***	0.60 ***	0.57 ***	**0.83 *****	NS	NS	NS
Day	NS	NS	NS	NS	NS	NS	NS
WC	NS	NS	NS	NS	NS	NS	0.38 **
Soil T (5cm)	NS	NS	-0.37 *	NS	NS	0.45 **	NS
Soil T (10cm)	NS	-0.45 **	NS	-0.45 *	0.40 *	NS	NS
Clay	NS	0.45 **	NS	NS	**0.77 *****	NS	-0.39 *

* p<0.1, ** p<0.05, *** p<0.01; NS, No Significant.

In Figure 7.3 we summarized all the data used in the correlation analysis.

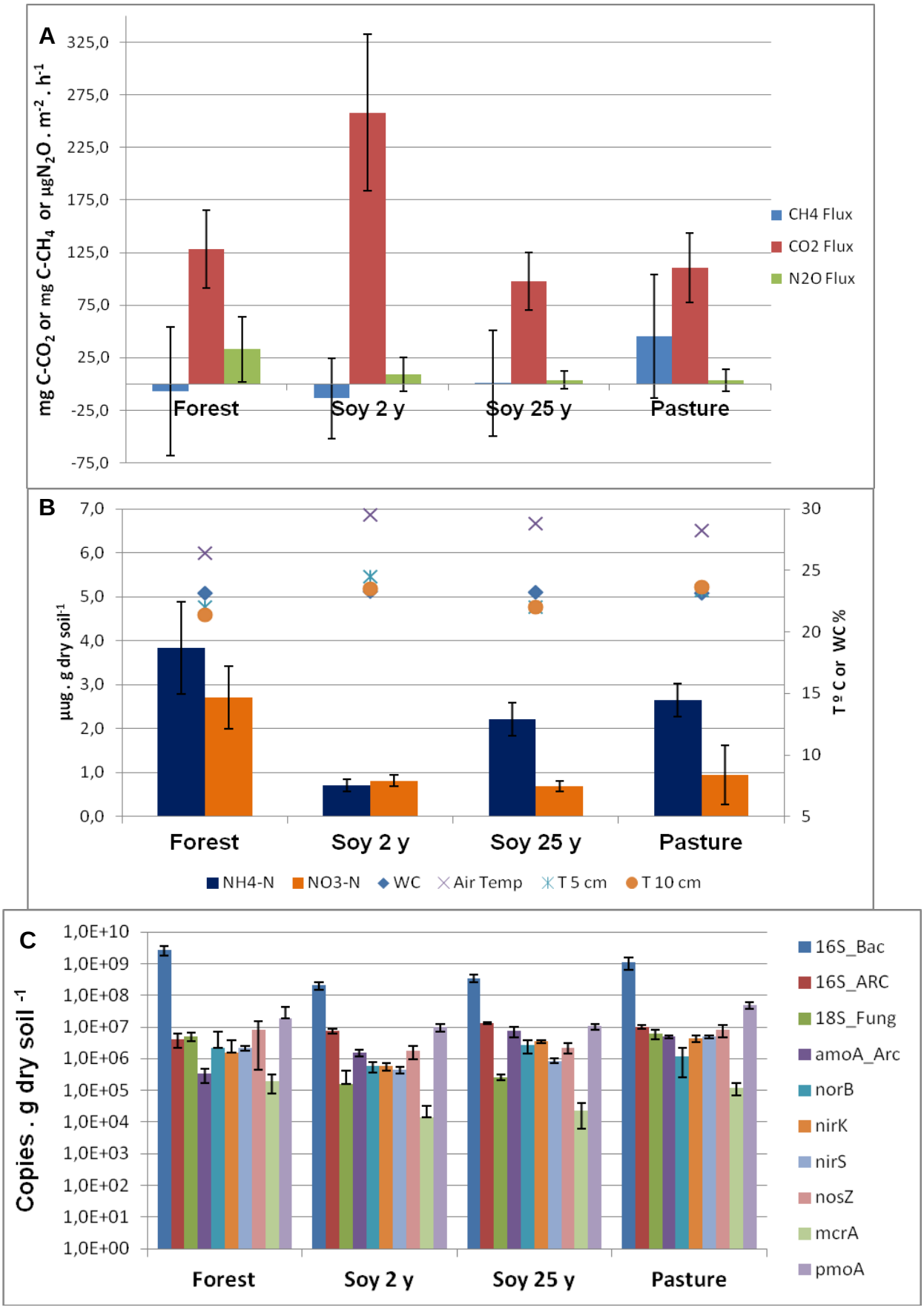

Figure 7.3 – GHG fluxes (A), selected attributes (B) and genes abundances (C)

7.4 Discussion

7.4.1 Environmental and soil chemical attributes

Since the sites were placed in the same edapho-climatic region, no changes were observed in relation to water content and air temperature. Little difference was observed in the texture of the soils. Clay content in soil of the pasture was slightly higher than the other sites, but all sites were classified as clay texture. Since the texture and precipitation were similar for the sites within the same region, water content was not significant differently among the samples (Table 7.2).

Soil temperature presented significant differences among the areas. It was slightly higher in pasture and soybean sites. The presence of higher and complex vegetation and a great litter protection made the soil in forest less sensitive to heating. Old pasture and the no-till system presented higher litter coverage than the new soybean area, still under conventional management, which caused the new soybean site to have intermediate soil temperatures. It was not observed statistical difference between the forest and the pasture.

We did not quantify the litter amount over the Forest soils, but it can be visually observed that the litter is higher than at the other sites (Figure 7.1). Litter fall in Amazonian Forests ranges from 0.6 to 0.8 $kg.m^2.year^{-1}$ (BRANDO et al., 2008). This fact causes several alterations in several soil attributes, as increasing C content in soil. The forest had the highest concentration of total C and organic matter. In addition, ammonium, nitrate and total N concentrations were highest in the forest due to higher nitrogen cycling in forest systems.

In our survey in 2009 we detected higher concentration of nitrate than ammonium in the soybean fields. In this new survey it was the opposite, higher ammonium than nitrate. This trend is probably due to the fact that the soybean field was surveyed in stage R3. Usually it is expected that higher nitrate concentration in the soybean field be found, but on the R3 stage, soybean is in the stage of legume formation and all available nitrogen is transferred to the legumes. Despite that soybean is a nitrogen-fixing species, and in Brazil *Bradyrhizobium japonicum* and *B. elkani* inoculation is the only N-fertilization management, due to the high demand in R3, nitrogen in the soil can be absorbed by the plant (PEREIRA et al., 2011).

Additionally, soybean has a preference for ammonium acquisition from the soil when pH is near 7.0, but a preference for nitrate due to the proton equilibrium when pH tends to 3.0 (HAWKINS; ROBBINS, 2010). Since the pH of the areas are between, it is probable the preference of soybean per nitrate, explaining the data.

Liming and fertilizers were responsible for the most soil parameters differences among the land uses. Liming increased the pH, Ca and Mg of the agriculture and pasture sites. Fertilizers increased the levels of K and P, which are important nutrients for the crops. Since *Brachiaria* is tolerant to low pH and low fertility, farmers do not take the same care in these practices as with the agricultural areas, and this difference can be observed among the land uses. The higher fertility of the agricultural sites is clear because the soil management to crop and pasture production affected the soil parameters (CARVALHO et al., 2010).

7.4.2 Gene abundance of the process-related genes

We did not detect statistical differences among the genes and land uses with the November survey which indicates that our hypothesis that the drought period and fallow cause same the effect on all land uses is feasible (Figure 7.2).

Compared to the survey performed on January, several differences were observed. Bacterial abundance was lower in soybean fields than the other land uses. This result may be a reflection of the pesticides application during the soybean growth. Pasture presented values similar to forest and it is known that conversion of forest to pasture does not reduce microbial diversity (JESUS et al., 2007). Archaea was more abundant in the soybean field than the Forest, and intermediate in Pasture. The same trend was observed in Chapter 3. Several parameters change from Forest to Soybean and Pasture, so it is speculative address which changes could result in higher Archaea abundance. It can be related with the higher pH, nutrient availability or root exsudates. Furtheres studies must be done to clarify these trends. Fungi were lower in soybean, which can be justified by the use of fungicides during the soybean management.

The gene *amo*A was higher in the older soybean field, which may be a consequence of the higher ammonium availability during the soybean cultivation. Soybean is supplied by nitrogen fixation, and as described before, has preference for nitrate in pH lower than 7.0. It was a relative surprise to see a higher community of

*amo*A, but a lower concentration of nitrate. As it was described before, in stage R3, soybean has a high acquisition of nitrate from soil, so the demand of the soybean is probably higher than the conversion of ammonium by the amonifiers community. Or the plant and management offer some suppression in the processes, as occurs for *Brachiaria* in the nitrification process (SUBBARAO et al., 2009).

The denitrifiers were quantified from all sites. The denitrifiers were higher in abundance in the forest and pasture sites, even though the production of nitrous oxide is highest in the forest site. *Brachiaria* can be an inhibitor of nitrification and *Brachiaria* fields presented lower nitrate concentration, what also may explain lower nitrous oxide emission. Its presence may explain why the process is not as high in the forest even with high gene abundance (SUBBARAO et al., 2009).

Methanogenic gene abundance was higher in the forest rather than the pasture, but methane production was more evident in the pasture. This trend may be related to the higher soil bulk density of the pasture from the cow grazing, resulting in anaerobic sites in the soil. In addition, cow excrements, which are more easily degradable than litter from the forest, may serve as a nutrient source to the microbes (CARVALHO et al., 2010). The quantity of methanotrophs, represented by the detection of the gene *pmo*A, was equal in all the land uses.

The ratio among genes was variable within land uses. Most of the ratios follow the same trends previously discussed for the individual genes.

The general trend is that microbial abundances of the pasture were more similar with the forest, while soybean fields presented slightly more abundance. Other studies showed higher microbial activity in pastures, comparable or superior to the forest sites (JESUS et al., 2007).

Despite the interpretation of this survey on January, the no significant differences observed for the November survey show that gene abundance in soil is influenced by the climate and also by the phenological stage of plants, as discussed for soybean in R3. Therefore, a larger scale temporal survey must be conducted to study how genes and communities change over time.

7.4.3 GHG measurements

Our data is in accordance with several studies conducted in Brazil. In general, the main trend is that methane emission in the pasture sites is, in the average, approximate to zero C-CH_4 mg, as balance of the positive and negative flux observed, due to the high spatial variability. In soybean fields and forests, usually is observed methane sinks (CARVALHO et al., 2010).

The soybean field in its second year presented higher carbon dioxide emission. Similar emission levels in old soybean, pasture and forest sites were detected as well as with other studies (CARVALHO et al., 2010).

7.4.4 Correlations between genes and processes

Several correlations were observed for carbon, nitrogen and the GHGs measurements in this study. Total C and N were correlated with each other and also with ammonium, nitrate, methane and nitrous oxide. This trend was expected because these compounds are related with each other (MOREIRA; SIQUEIRA, 2006; SIQUEIRA et al., 2011).

Significant correlations among bacterial and denitrifiers genes, C and N forms and nitrous oxide emission were identified. Bacterial abundance was also correlated with C, N, nitrate and nitrous oxide emission. These correlations indicate that qPCR was a good technique to detect the microbiological processes present in the soil (MORALES et al., 2010).

The method for the quantification of genes mcrA and norB needs further optimization because most of the samples yielded amounts below the detection limit. Therefore, the reported average in this study would be not that all reliable. Both primer sets were designed from other studies, probably with sequences that do not reflect the reality of our study sites.

In contrast to results from Morales et al. (2010), we did not find any correlation between the subtraction of *nir*S copy number with *nos*Z, or other combinations, including *nirK*, with nitrous oxide emission (Figure 7.4). However, we found good correlation between *nir*K and *nos*Z, which was expected because these genes are

usually located in the same operon (RICHARDSON et al., 2009; JONES et al., 2008). Therefore, we disagree with the notion that subtraction of *nir*K or *nir*S with *nos*Z as presented in the study by Morales et al. (2010) is appropriate.

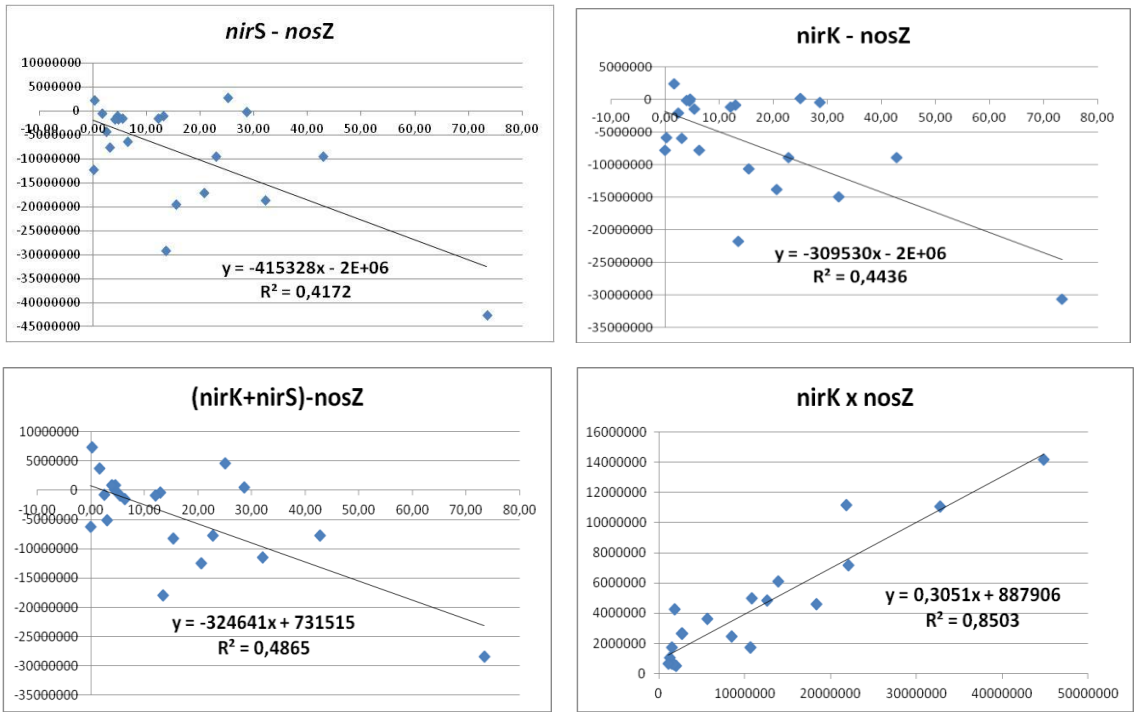

Figure 7.4 – Linear correlations between *nir*S-*nos*Z with N_2O (up left); between *nir*K-*nos*Z with N_2O (up right); (*nir*K+*nir*S)-*nos*Z with N_2O (bottom left); and correlation between *nir*K and *nos*Z (bottom right)

Other correlations that we found are in accordance with Richardson et al. (2009). We found correlation between nitrate, nitrous oxide, pH and C. The only correlation that we do not find was between Cu and the nitrous oxide processes-related factors. Cu is not only needed for the Nos protein, but also for Nor, Nir and several other bacterial proteins (THROBÄCK et al., 2004; ZUMFT, 2005). Additionally, it is also a nutrient for plants, so we do not believe that Cu will be a limiting factor for bacteria, since Cu deficiency is suppose to be noticed first in plants that use much higher amounts than bacteria (MARSCHNER, 1995).

We have showed experimentally, that significant correlations among the evolved genes and processes exist. One of the main reasons is that most of these genes have constitutive expression and are related to anaerobic processes, that means environmental survival. It is clear for *mcr*A, since the expression is obligatory

for the archea survive in the anaerobic conditions. Similar is the idea for the denitrification genes. Aerobics and anaerobic shifts can occurs rapidly in soil, as a strong rain after a dry period, so having the protein cluster ready to use is essential for survey. Despite this constitutive expression need, the expression is also regulated for nitrate levels and other factors (ZUMPFT, 2005). It is still speculative affirmation about correlation about genes and protein levels in the environment, since most of the studies were conduct in vitro with isolates (RICHARDSON et al., 2009). But since we detected correlations between gene abundance and the processes, it is expected significant correlation between genes, cell number and protein. Further improvements of proteomics and in situ identification of the bacteria techniques in soil, will provide us a better understand of the processes.

7.4.5 qPCR improvement

A concern that is little discussed in most of the qPCR papers in soil is the need of technique improvement. Most of papers work with a few genes, probably because the elimination of bad results with some of the tested primers. The qPCR efficiency and limit detections are an interaction of nucleic acid quality, gene abundance in the sample and primer design (SALEH-LAKHA et al., 2011).

We noticed that for genes of higher abundance and primers of higher specificity, as 16S rRNA, inhibition effect is very little, and qPCR efficiency is generally > 90%. For other genes that primers are too specific and the abundance is lower, such *nor*B or *mcr*A, the effects of inhibition is amplified. When the limit or abundance is low, increasing sample would solve the problem. But increasing DNA amount in the PCR reaction means increase the inhibitors concentrations, what is prejudicial to the reaction.

For the low abundance genes, obviously, DNA quantity matters. We noticed that different protocols offer different extraction yield of the samples. And increasing purity, reduce yield. This is a big challenge for qPCR, since the results are in g .dry soil, a difference of 10 times in the extraction yield, can consequently reflect in an error up to 10 times in the quantification (Appendix A).

In matter of the fact, both, DNA quality and quantity matters, and no protocol are still perfect for the tropical soil samples. We could reduce the inhibition effect using inhibitors resistant enzyme and T4 Gene 32 protein, but even that, some underestimation could have occurred (Appendix B).

Other problem is representatively of the sample. Usually the extraction protocols use 0.25 g, what means that the soil sample may be under represented. For example, *mcr*A is a gene present in anaerobic Archaea, which may live in anaerobic micro sites in the soil. Sieving and homogenizing the samples may disrupt these sites, killing the organisms and diluting the DNA in a strong degradable environment. Further studies may keep in mind keeping the soil structure, but using higher amount of soils, as nitrate analysis that use 4-20 g of soil, what makes the sample much more representative.

The other challenge is primer design. There is a paradox that conservative primers are more specific and usually result in better amplification, but lower diversity. A less conservative primer would access a wider range of diversity, but the possibility of no specific amplification and smears is higher.

Other problem is that most of the primers were design based on a few isolates, as *nor*B, which can not represent all the community in the soil (BRAKER; TIEDJE, 2003; DANDIE et al., 2006). Testing *nor*B primers in our samples, only the primer of Dandie et al. (2006) presented good amplification, resulting in specific bands and sharp melting curve peaks. But the copy number of *nor*B in soil was always lower than *nir*K, *nir*S and *nos*Z, usually located in the same operon (JONES et al., 2008). This fact is a stronger indicator that the *nor*B primer is too group specific, since it would be expected correlation among these genes. Additionally, Dandie et al. (2006) could not amplify *nor*B for several denitrifier species, showing the primer specificity.

In our study, as in Morales et al. (2006), we found good correlation between genes and processes, especially because we studied really contrasting environments. For further studies with less contrasting environments, it is clear that optimization of all these discussed factors will be fundamental in finding correlations.

New enforces has been made for soil DNA extraction improvement and primer design can be huge improved with new metagenomics studies that allows to access a more representative amount of sequences.

Metagenomics, which is primer independent, looks more promising for a real access to the communities in the samples. But price and technique details are still limiting factors, since no study has completely sequenced a soil sample yet.

Nowadays, qPCR is a cheap and promising technique, which allows integrating representative microbiological parameters to the C and N cycles, providing potential to be used in further models.

7.5 Conclusions

Our chemical data are in accordance with other studies showing differences among the land uses in soil chemical parameters such as GHG emissions.

The thesis hypothesis was confirmed, showing significant correlation among the genes and processes.

We found significant correlations between the denitrifiers related genes and nitrous oxide emission.

The primers used to detect *nor*B and *mcr*A did not present good amplification and need further optimization.

Improvement in the qPCR analysis would result in higher correlation with the processes. For this is need improvement in DNA extraction and primer design.

8. GENERAL CONCLUSIONS

***"Hypothesis**: Land use change from natural environment to agriculture and cattle raising results in physical, chemical, and biological soil changes that cause alterations in C, N and GGE. Soil microbial groups abundance, accessed by qPCR, may be a good indicator of these changes and of GGE. "*

The hypothesis was confirmed:

- The main effects observed in soils involved in land use change were chemical changes, such as changes in pH, and in C, N, P, Ca and Mg concentrations. Microbial structures also changed, and correlated with chemical changes in three independent field studies.

- Changes in the abundance of genes involved in soil biogeochemical cycles correlated with changes in the respective processes, showing that gene quantitation corroborates important microbial information for the understanding of biogeochemical cycles. The change in gene quantity is a sensitivity indicator for land use change.

- A larger temporal and spatial analysis is needed to validate gene abundance as a good indicator of greenhouse gas emissions.

***"General objective**: To analyze microbial functional abundance by examining C, N and GHG-related genes in soils under different land use type in Southwestern Amazon, and establishing a relationship with these biogeochemical processes. "*

- Based on this general objective, a model was constructed (Figure 8.1).

- In the model all functions of the C and N cycle were integrated with those of the GHG. This should introduce gene abundance as an indicator of the biogeochemical processes.

The specific relationships observed are discussed below with each specific objective.

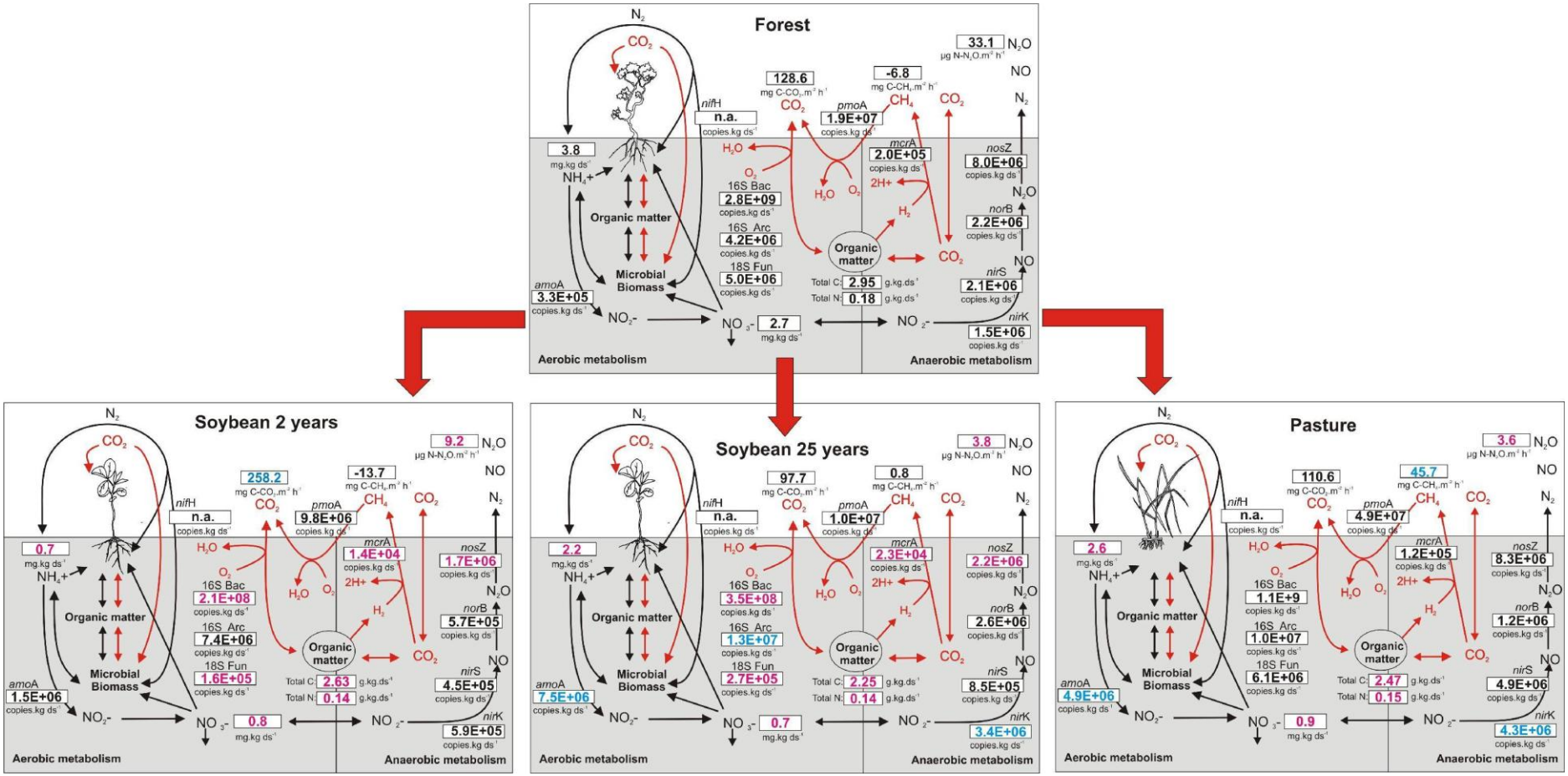

Figure 8.1 – Integrated model for land use change in the soils of Southwestern Amazonia. The abundance of soil microbial genes involved in biogeochemical cycles of C and N are shown together with their respective end products. The Forest was set as the reference value representing an unchanged control. Values that change with land use change from forest to Soybean or Pasture are indicated in color. Increases in blue, and decreases in pink

"Specific objectives"

" a) Characterize how the soil microbial communities shift with land use change from Cerrado and Amazon Forest to agriculture and pasture by correlating soil chemical attributes with T-RFLP (16S rRNA) and qPCR".

- The main effects observed in soils involved in land use change were chemical changes, such as changes in pH, and in C, N, P, Ca and Mg concentrations. Microbial structures also changed, but no loss in diversity was observed; bacterial richness was maintained.

" b) Case study to evaluate land use change, from Cerrado vegetation to agriculture and pasture, impact on C, N stocks and microbial attributes, biomass C and qPCR of the 16S rRNA gene in Bacteria and Archaea and the 18S rRNA gene in Fungi."

- Land use change from Cerrado to agriculture and pasture affected certain, but not all, soil chemical characteristics. For example, when the land use changed from Cerrado to agriculture and pasture, the total nitrogen content in the soil increased. This change may be attributed to management practices since manure additions, fertilization to successional crops, and N fixation in soybeans added N to the soil. Also C stocks increased, especially due to increase in the bulk density. Liming and fertilizers, obviously also changed additional chemical parameters such as P, Ca, Mg, K, and pH.

- Some of these soil parameters either correlated weakly or not at all with a change in land use, namely microbial biomass, while the abundance of Archaea and Fungi was correlated with land use change. Although these variable results suggest that further study will be needed, we can conclude that there is a significant change in soil chemistry and microbiota when land use changes from Cerrado to agriculture and pasture.

" c) To test the plant effect of soybean and Brachiaria in mesocosms, evaluating the fluxes of GHG (CO_2, CH_4, N_2O, by GC) and correlating with soil chemical attributes and qPCR of processes related genes (norB, nosZ, mcrA and pmoA)."

- The mesocosms studies indicated low influence of plants on the microbial community structures. However, some changes were significant. Plant presence changed the soil nitrate concentration due to plant nutrient uptake. The changes in nitrate were correlated with denitrifier abundance and nitrous

oxide emissions. Higher bacterial abundance was associated with plant presence in the mesocosms.

- Comparing the changes observed in the other chapters, we concluded that a change in land use, rather than in plant species, promote the most significant alterations in the microbial structures and processes.

“ d) Evaluation of GHG fluxes (CO_2, CH_4, N_2O, by GC) and correlating with soil chemical attributes and qPCR of processes related genes (amoA, nirK, nirS, norB, nosZ, mcrA and pmoA) in soil samples surveyed in situ from Amazonian Forest and conversion to pasture and agriculture.”

- The field study showed a positive correlation between total C and N, gene abundance, and GHG production.

- During the survey period, the Forest showed a higher microbial activity, resulting in higher nitrate availability and N_2O emissions, which correlated with higher gene abundance of the involved process genes.

- Nitrate and N_2O emissions were lower in agriculture and pasture. The CO_2 emission was higher in Soybean in its 2nd year. Forest and Soybean 2nd year were a sink of CH_4, while pasture acted as a source.

“ e) To find and discuss correlations among genes abundance and soil chemical parameters related to the C, N and GHG cycles.”

- Significant correlations among bacterial and denitrifier genes, forms of C and N, and nitrous oxide emissions were identified. Bacterial abundance was also correlated with C, N, nitrate and nitrous oxide emissions. These correlations indicate that gene abundance is a good indicator of the processes.

- The analysis of gases varied throughout days according to environmental factors, such as temperature and humidity, but the microbial community, represented by gene abundance, was more stable.

- Accessing the genes of the groups of microorganisms that produce the gases (denitrifying, methanogenic), may allow access to the potential of gas production for each soil. Bigger temporal and spatial survey may provide parameters to develop such models.

Final considerations:

- One important step to trace strategies to mitigate greenhouse gases is better knowledge of the organisms that produce the gases. This thesis provided information about the use of molecular techniques to better understand the ecology of these microorganisms.

- Our results validated the use of gene abundance via qPCR to better understand the processes, but a bigger temporal and spatial analysis is needed to infer statements on the process dynamic due to land use change.

- Improving the proposed model and integrating with physical data, would improve C, N and GHG modeling accuracy. Most of the available models use poor microbial parameters, and this thesis showed that gene abundance could be a good parameter to be included in the estimation models.

The contribution of this thesis is an integrated model, that contains quantitative microbial parameters, which present strong correlations with the biogeochemical processes, giving insights how land use change impacts the Southwestern Amazonian agricultural frontier.

Studying land use as an integrated relation between chemical and biological parameters that respond to environmental changes, such as rain and temperature, would provide a more precise overview of the processes.

A better overview of the processes would provide a more accurate identification of management practices that improve natural resources conservation and optimize agricultural practices, along with key points for sustainability.

REFERENCES

AMANN, R. I.; LUDWIG, W.; SCHLEIFER, K. H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation**. Microbiological Reviews**, Washington, DC, v. 59, p. 143–169, 1995.

BACH, H.-J.; TOMANOVA, J.; SCHLOTER, M.; MUNCH, J. C. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. **Journal of Microbiological Methods**, Amsterdam, v. 49, p. 235–245, 2002.

BARONA, E.; RAMANKUTTY, N.; HYMAN, G.; COOMES, O. The role of pasture and soybean in deforestation of the Brazilian Amazon. **Environmental Research Letters**, Bristol, v. 5, 9 p. 2010. doi:10.1088/1748-9326/5/2/024002.

BATLLE-BAYER, L.; BATJES, N. H.; BINDRABAN, P. S. Changes in organic carbon stocks upon land use conversion in the Brazilian Cerrado: A review. **Agriculture, Ecosystems and Environment**, Amsterdam, v. 137, p. 47-58, 2010.

BAYER, C.; MEILNICZUK, J. Dinâmica e função da matéria orgânica. In: SANTOS, G. A.; CAMARGO, F. A. O. (Ed.). **Fundamentos da matéria orgânica do solo:** ecossistemas tropicais e subtropicais. Porto Alegre: Gênesis, 1999. p. 1-26.

BRAKER, G.; TIEDJE, J. M. Nitric oxide reductase (norB) genes from pure cultures and environmental samples. **Applied and Environmental Microbiology***,* Baltimore, v. 69, p. 3476–3483, 2003.

BRANDO, P. M.; NEPSTAD, D. C.; DAVIDSON, E. A.; TRUMBORE, S. E.; RAY, D.; CAMARGO, P. Drought effects on litterfall, wood production and belowground carbon cycling in an Amazon forest: results of a throughfall reduction experiment. **Philosophical Transactions of the Royal Society B**, London, v. 363, p. 1839–1848, 2008.

BRASIL. Ministério da Agricultura e do Abastecimento. Brasília, DF, 2011. Available at: http://www.agricultura.gov.br/. Accessed: Jul. 11, 2011.

BRASIL. Ministério da Ciência e Tecnologia. **Comunicação Nacional Inicial do Brasil à Convenção-Quadro das Nações Unidas sobre Mudança do Clima**. Coordenação-Geral de Mudanças Globais de Clima. Brasília, DF, 2004. 74 p.

BREMER, C.; BRAKER, G.; MATTHIES, D.; REUTER, A.; ENGELS, C.; CONRAD, R. Nitric oxide reductase (norB) genes from pure cultures and environmental samples. Applied and Environmental Microbiology, Baltimore, v. 73, p. 6876–6884, 2007.

CANFIELD, D. E.; GLAZER, A. N.; FALKOWSKI, P. G. The evolution and future of earth's nitrogen cycle. **Science**, Washington, DC, v. 330, n. 6001, p. 192-196, 2010.

CAPRA, J.; HENDERSON, H. **Qualitative growth:** A conceptual framework for finding solutions to our current crisis that are economically sound, ecologically sustainable, and socially just. Berkeley: Institute of Chartered Accountants in England & Wales, 2009. Available at: http://www.fritjofcapra.net/articles100709.html Accessed: Oct. 10, 2011.

CARVALHO, J. L. **Dinâmica do carbono e fluxo de gases do efeito estufa em sistemas de integração lavoura-pecuária na Amazônia e no Cerrado**. 2010. 141 p. Tese (Doutorado em Solos e Nutrição de Plantas) – Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, 2010.

CARVALHO, J. L. N.; CERRI, C. E. P.; CERRI, C. C.; FEIGL, B. J.; PICCOLO, M. C.; GODINHO, V.; HERPIN, U. Changes of chemical properties in an Oxisol after clearing of native Cerrado vegetation for agricultural use in Vilhena, Rondonia State, Brazil. **Soil and Tillage Research**, Amsterdam, v. 96, p. 95-102, 2007.

CARVALHO, J. L. N.; CERRI, C. E. P.; FEIGL, B. J.; PICCOLO, M. C.; GODINHO, V. P.; HERPIN, U.; CERRI, C. C. Conversion of Cerrado into agricultural land in the south-western Amazon: carbon stocks and soil fertility. **Scientia Agricola**, Piracicaba, SP, v. 66, p. 233-241, 2009.

CENCIANI, K.; MAZZETTO, A. M.; LAMMEL, D. R.; FRACETTO, F. J.; FRACETTO, G. G. M.; FRAZAO, L.; CERRI, C. C.; FEIGL. B. Genetic and functional diversities of microbial communities in Amazonian soils under different land uses and cultivation. In: MATOVIC, D. (Ed.). **Biomass -** detection, production and usage. Rijeka:Croacia: INTECH, 2011.

CENTRO DE ESTUDOS AVANÇADOS EM ECONOMIA APLICADA – CEPEA. Available at: http://www.cepea.esalq.usp.br. Accessed: Aug. 25, 2007.

CERRI, C. C.; BERNOUX, M.; CERRI, C. E. P.; FELLER, C. Carbon cycling and sequestration opportunities in South America: the case of Brazil. **Soil Use and Management**, New York, v. 20, n. special, p. 248-254, 2004.

CLAESSON, M. J.; WANG, J.; O'SULLIVAN, O.; GREENE-DINIZ, R.; COLE, J. R.; ROSS, R. P.; O'TOOLE, P. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. **Nucleic Acids Research**, Oxford, v. 38, n. 22, 13 p., 2010. doi: 10.1093/nar/gkq873.

COHAN, F. M. What are bacterial species? **Annual Review of Microbiology**, Palo Alto, v. 56, p. 457-487, 2002.

D´ANDRÉA, A. F.; SILVA, M. L. N.; CURI, N.; GUILHERME, L. R. G. Carbon and nitrogen storage, and inorganic nitrogen forms in a soil under different management systems. **Pesquisa Agropecuária Brasileira**, Brasília, DF, v. 39, p. 179–186, 2004.

DANDIE, C. E.; BURTON, D. L.; ZEBARTH, B. J.; TREVORS, J. T.; GOYER C. Analysis of denitrification genes and comparison of *nosZ*, *cnorB* and 16S rDNA from culturable denitrifying bacteria in potato cropping systems. **Systematic and Applied Microbiology**, Stuttgart, v. 30, p. 128-138, 2007.

DANDIE, C. E.; MILLER, M. N.; BURTON, D. L.; ZEBARTH, B. J.; TREVOR, J. T.; GOYER, C. Nitric oxide reductase-targeted real-time pcr quantification of denitrifier populations in soil. **Applied and Environmental Microbiology***,* Baltimore, v. 73, n. 13, p. 4250–4258, 2007.

DHILLON, A.; LEVER, M.; LLOYD, K. G.; ALBERT, D. B.; SOGIN, M. L.; TESKE, A. Methanogen diversity evidenced by molecular characterization of methyl coenzyme M reductase A (mcrA) genes in hydrothermal sediments of the Guaymas Basin. **Applied and Environmental Microbiology***,* Baltimore, v. 71, p. 4592-4601, 2005.

DÖLKEN, L.; SCHÜLER, F*.* Quantitative detection of positive cell by real-time quantitative PCR using fluorogenic probes. **Biotechniques**, Natick, v. 6, p. 1058-1064, 1998.

EMBRAPA. **Banco de dados climáticos do Brasil**. Campinas, SP: Embrapa Monitoramento por Satélite, 2003. Available at: http://www.bdclima.cnpm.embrapa.br/resultados. Accessed: Nov. 12, 2009.

FEIGL, B. J.; MELILLO, J. M.; CERRI, C. C. Changes in the origin and quality of soil organic matter after pasture introduction in Rondonia. **Plant and Soil**, Dordrecht, v. 175, p. 21-29, 1995.

FOLEY, J. A.; DEFRIES, R.; ASNER, G. P.; BARFORD, C.; BONAN, G.; CARPENTER, S. R.; CHAPIN, F. S.; COE, M. T.; DAILY, G.; GIBBS, H. K.; HELKOWSKI, J. H.; HOLLOWAY, T.; HOWARD, E. A.; KUCHARIK, C. J.; MONFREDA, C.; PATZ, J. A.; PRENTICE, I. C.; RAMANKUTTY, N.; SNYDER, P. K. Global consequences of land use. **Science**, Washington, DC, v. 309, p. 570-574, 2005.

FRAZÃO, L. A.; CARVALHO, J. L. N.; MAZZETTO, A. M.; FRACETTO, F. J. C.; CENCIANI, K.; FEIGL, B. J.; CERRI, C. C. Soil microbial biomass under native cerrado and its changes after the pasture and annual crops introduction. In: ATAZADEH, I. (Ed.). **Biomass and remote sensing of biomass**. Rijeka, Croatia: InTech, 2011. chap. 6, p. 87-110.

FRAZÂO, L. A.; PICCOLO, M. D. C.; FEIGL, B. J.; CERRI, C. C.; CERRI, C. E. P. Inorganic nitrogen, microbial biomass and microbial activity of a sandy Brazilian Cerrado soil under different land uses. **Agriculture, Ecosystems and Environment**, Amsterdam, v. 135, p. 161-167, 2010.

FREITAG, T. E.; PROSSER, J. I. Correlation of methane production and functional gene transcriptional activity in a peat soil. **Applied and Environmental Microbiology***,* Baltimore, v. 75, n. 21, p. 6679-6687, 2009.

FREITAS, P. L.; BLANCANEAUX, P.; GAVINELLI, E.; LARRE-LARROUY, M. C.; FELLER, C. Nível e natureza do estoque orgânico de Latossolos sob diferentes sistemas de uso e manejo. **Pesquisa Agropecuária Brasileira**, Brasília, DF, v. 35, p. 157-170, 2000.

FUJISAKA, S.; CATILLA, C.; ESCOBAR, G.; RODRIGUES, V.; VENEKLAAS, E. J.; THOMAS, R.; FISHER, M. The effects of forest conversion on annual crops and pastures: Estimates of carbon emissions and plant species loss in a Brazilian Amazon colony. **Agriculture, Ecosystems and Environment**, Amsterdam, v. 69, p. 17-26, 1998.

GROSSMAN, J. M.; O'NEILL, B.; TSAI, S. M.; LIANG, B.; NEVES, E.; LEHMANN, J.; THIES, E. J. Amazonian anthrosols support similar microbial communities that differ distinctly from those extant in adjacent, unmodified soils of the same mineralogy. **Microbial Ecology**, New York, NY, v. 60, n. 1, p. 192-205, 2010.

HALLIN, S.; LINDGREN, P.-E. PCR detection of genes encoding nitrite reductase in denitrifing bacteria. **Applied and Environmental Microbiology***,* Baltimore, v. 65, p. 1652–1657, 1999.

HALLIN, S.; JONES, C. M.; SCHLOTER, M. PHILIPPOT, L. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. **The ISME Journal**, New York, NY, v. 3, p. 597-605, 2009.

HAWKINS, B. J.; ROBBINS, S. pH affects ammonium, nitrate and proton fluxes in theapical region of conifer and soybean roots**. Physiologia Plantarum**, Kobenhavn, v. 138, p. 238–247, 2010.

HEID, C. A.; STEVENS, J. Real time quantitative PCR. **Genome Research**, Woodbury, v. 6, p. 986-994, 1996.

HENRY, S.; BRU, D. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. **Applied and Environmental Microbiology**, Baltimore, v. 72, n. 8, p. 5181-5189, 2006.

HOU, Y.; ZHANG, H.; MIRANDA, L.; LIN, S. Serious overestimation in quantitative PCR by circular (Supercoiled) plasmid standard: microalgal *pcna* as the model gene. **PLoS ONE**, San Francisco, v. 5, n. 3, p. e9545, 2010.

INNEREBNER, G.; KNAPP, B. Traceability of ammonia-oxidizing bacteria in compost-treated soils. **Soil Biology and Biochemistry**, Oxford, v. 38, n. 5, p. 1092-1100, 2006.

INSTITUTO BRASILEIRO DE FLORESTAS – IBF. **Biomas do Brasil**. Curitiba, PR, 2011. Available at: http://www.ibflorestas.org.br/en/bioma-cerrado.html Accessed: Oct. 10 2011.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA – IBGE. Rio de Janeiro, RJ, 2011. Available at: http://www.ibge.gov.br/. Accessed: Nov. 12, 2010.

INSTITUTO NACIONAL DE PESQUISAS ESPACIAIS – INPE. **Amazônia**: desflorestamento 2002-2003. São José dos Campos, SP, 2004.

INTERGOVERNMENTAL PANEL ON CLIMATE CHANGES – IPCC. Available at: http://www.ipcc.ch/. Accessed: Aug. 25 2007.

ISLAM, T.; JENSEN, S.; REIGSTAD, L. J.; LARSEN, O.; BIRKELAND, N. Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum. **Proceedings of the National Academy of Sciences of the USA**, Washington, DC, v. 105, n. 1, p. 300-304, 2008.

JESUS, E. C.; MARSCH, T. L.; TIEDJE, J. M.; MOREIRA, F. M. S. Changes in land use alter the structure of bacterial communities in Western Amazon soils. **The ISME Journal**, New York, NY, v. 3, p. 1004-1011, 2009.

JONES, C. M.; STRES, M.; ROSENQUIST, M.; HALLIN, S. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. **Molecular Biology and Evolution**, Chicago, v. 25, n. 9, p. 1955–1966, 2008.

KANG, Y. J.; CHENG, J.; MEI, L. J.; HU, J.; PIAO, Z.; YIN, S. X. Multiple copies of 16S rRNA gene affect the restriction patterns and DGGE profile revealed by analysis of genome database. **Microbiology**, Moscow, v. 79, n. 5, p. 655-662, 2010.

KERMEKCHIEV, M. B.; KIRILOVA, L. I.; VAIL, E. E.; BARNES, W. M. Mutants of Taq DNA Polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. **Nucleic Acids Research**, Oxford, v. 37, n. 5, e40, 2009. doi: 10.1093/nar/gkn1055.

KERR, R. A. How hot will the greenhouse world be? **Science**, Washington, DC, v. 309, n. 5731, p. 100, 2005.

KNORR, W.; PRENTICE, I. C.; HOUSE, J. I.; HOLLAND, E. A. Long-term sensitivity of soil carbon turnover to warming. **Nature**, London, v. 433, p. 298-301, 2005.

LAL, R. Global potential of soil carbon sequestration to mitigate the greenhouse effect. **Critical Reviews in Plant Sciences,** Boca Raton, v. 22, p. 151-184, 2003.

LEININGER, S.; URICH, T. Archaea predominate among ammonia-oxidizing prokaryotes in soils. **Nature**, London, v. 442, n. 7104, p. 806-809, 2006.

LEPS, J.; SMILAUER, P. **Multivariate analysis of ecological data using CANOCO.** Cambridge: Cambridge University Press, 2003. 269 p.

LIU, W. T.; MARSH, T. L.; CHENG, H.; FORNEY, L. J. Characterization of microbial diversity by determinng terminal restriction fragment length polymorphism of genes encoding 16S rRNA. **Applied and Environmental Microbiology,** Baltimore, v. 63, p. 4516-4522, 1997.

MARENGO, J. A. **Mudanças climáticas globais e seus efeitos sobre a biodiversidade** - Caracterização do clima atual e definição das alterações climáticas para o território brasileiro ao longo do Século XXI. Brasília, DF, Ministério do Meio Ambiente, 2006. 212 p. (Série Biodiversidade, 26).

MARSCHNER, H. **Mineral nutrition of higher plants.** 2. ed. London: Academic Press, 1995.

MCDONALD, I. R.; BODROSSY, L.; CHEN, Y.; MURRELL, J. C. Molecular ecology techniques for the study of aerobic methanotrophs. **Applied and Environmental Microbiology**, Baltimore, v. 74, n. 5, p. 1305-1315, 2008.

MEIER, C. L.; BOWMAN, W. D. Links between plant litter chemistry, species diversity, and below-ground ecosystem function. **Proceedings of the National Academy of Sciences of the USA**, Washington, DC, v. 105, n. 50, p. 19780-19785, 2008.

MENDES, L. W.; TSAI, S. M. Estudo da diversidade microbiana em solos de Mangue, floresta e restinga através do T-RFLP (Terminal Restriction Fragment Length Polymorphism). In: SIMPÓSIO DOS PÓS-GRADUANDOS NO CENA/USP, 1., 2008, Piracicaba. **Resumos...** Piracicaba, SP: CENA/USP, 2008. 1 CD-ROM.

MORALES, S.; COSART, T.; HOLBEN, W. Bacterial gene abundances as indicators of greenhouse gas emission in soils. **The ISME Journal**, New York, NY, v. 4, p. 799–808, 2010.

MOREIRA, F. M. S.; SIQUEIRA, J. O. **Microbiologia e bioquímica do solo.** Lavras: Editora UFLA, 2006. 729 p.

MUNGAI, N. W.; MOTAVALLI, P. P. Litter quality effects on soil carbon and nitrogen dynamics in temperate alley cropping systems. **Applied Soil Ecology**, Amsterdam, v. 31, p. 32–42, 2006.

NAVARRETE, A. A.; CANNAVAN, F. S.; TAKETANI, R. G.; TSAI, S. M. A molecular survey of the diversity of microbial communities in different Amazonian agricultural model systems. **Diversity**, Bethesda, v. 2, p. 787-809, 2010.

NEILL, C.; MELILLO, J. M.; STEUDLER, P. A.; CERRI, C. C.; DEMORAES, J. F. L.; PICCOLO, M. C.; BRITO, M. Soil carbon and nitrogen stocks following forest clearing for pasture in the southwestern Brazilian Amazon. **Ecological Applications**, Ithaca, v. 7, n. 4, p. 1216-1225, 1997.

NELSON, D. W.; SOMMERS, L. E. Total carbon, organic carbon and organic matter. In: PAGE, A. L.; MILLER, R. H.; KEENEY, D. R. (Ed.). **Methods of soil analysis**. 2. ed. Madison: ASA, 1982. p. 539-579.

OLIVEIRA, O. C.; OLIVEIRA, I. P.; ALVES, B. J. R.; URQUIAGA, S.; BODDEY, R. M. Chemical and biological indicators of decline/degradation of Brachiaria pasture in the Brazilian Cerrado. **Agriculture, Ecosystems and Environment**, Amsterdam, v. 103, n. 2, p. 289-300, 2004.

PEREIRA, E. I. P. P.; CHUNG, H.; SCOWC, K.; SADOWKY, M. J.; KESSEL, C.; SIX, J. Soil nitrogen transformations under elevated atmospheric CO2 and O3 during the soybean growing season. **Environmental Pollution**, London, v. 159, p. 401-407, 2011.

PIMENTEL, D.; BAILEY, O.; KIM, P.; MULLANEY, E.; CALABRESE, J.; WALMAN, F.; NELSON, F.; YAO, X. **Will limits of the earth's resources control human numbers?** Ithaca, NY: Cornell University, 1999. Available at: http://dieoff.org/page174.htm. Accessed: Oct. 10, 2011.

RICHARDSON, D.; FELGATE, H.; WATMOUGH, N.; THOMSON, A.; BAGGS, E. Mitigating release of the potent greenhouse gas N2O from the nitrogen cycle-could enzymic regulation hold the key? **Trends in Biotechnology**, Cambridge, v. 27, n. 7, p. 388-397, 2009.

SALEH-LAKHA, S.; SHANNON, K. E.; GOYER, C.; TREVORS, J. T. Challenges in quantifying microbial gene expression in soil using quantitative reverse transcription real-time PCR. **Journal of Microbiological Methods**, Amsterdam, v. 85, n. 3, p. 239-243, 2011.

SANCHES, A. C.; MICHELLON, E.; ROESSING, A. C. Os limites de expansão da soja. **Revista Informe GEPEC**, Cascavel, PR, v. 9, n. 1, p. 1-21, 2005.

SIQUEIRA NETO, M.; PICCOLO, M. C.; COSTA JUNIOR, C. C.; CERRI, C. C.; BERNOUX, M. Emissão de gases do efeito estufa em diferentes usos da terra no bioma Cerrado. **Revista Brasileira de Ciência do Solo**, Viçosa, v. 35, n. 1, p. 63-76, 2011.

SIX, J.; OGLE, S. M.; BREIDT, F. J.; CONANT, R. T.; MOSIER, A. R.; PAUSTIAN, K. The potential to mitigate global warming with no-tillage management is only realized when practised in the long term. **Global Change Biology**, Oxford, v. 10, p. 155-160, 2004.

SPARLING, G.; ZHU, C. Evaluation and calibration of biochemical methods to measure microbial biomass C and N in soils from Western Australia. **Soil Biology and Biochemistry**, Oxford, v. 25, n. 12, p. 1793-1801, 1993.

STEINBERG, L. M.; REGAN, J. M. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. **Applied and Environmental Microbiology**, Baltimore, v. 74, n. 21, p. 6663–6671, 2008.

STEUDLER, P. A.; MELILLO, J. M.; BOWDEN, R.; CASTRO, M.; LUGO, A. The effects of natural and human disturbances on soil nitrogen dynamics and trace gas fluxes in a Puerto Rican wet forest. **Biotropica**, Washington, DC, v. 23, p. 356-363, 1991.

SUBBARAO, G. V.; NAKAHARA, K.; HURTADO, M. DEL P.; ONO, H.; MORETA, M. D. E.; SALCEDO, A. F.; YOSHIHASHI, A. T.; ISHIKAWA, T.; TITIAN, M.; OHNISHI, M.; YOSHIDA, M.; RONDÓN, M. A.; RAO, I. M.; LASCANO, C. E.; BERRY, W. L.; ITO, O. Evidence for biological nitrification inhibition in Brachiaria pastures. **Proceedings of the National Academy of Sciences of the USA**, Washington, DC, v. 106, n. 41, p. 17302-17307, 2009.

SVENNING, M.M., WARTIAINEN, I., HESTNES, A.G. & BINNERUP, S.J. Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. **FEMS Microbiology Ecology**, Amsterdam, v. 44, p. 347-354, 2003.

TEBBE, C. C.; VAHJEN, W. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. **Applied and Environmental Microbiology***,* Baltimore, v. 59, n. 8, p. 2657-2665, 1993.

THE ECONOMIST NEWSPAPER LIMITED. The miracle of the Cerrado. **The Economist**, St, Louis, MO, Aug. 26th 2010. Available at: http://www.economist.com/node/16886442Accessed 10 Oct. 2011, at:

THROBÄCK, I., N., ENWALL, K., JARVIS, A. HALLIN, S. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. **FEMS Microbiology Ecology**, Amsterdam, v. 49, p. 401–417, 2004.

TORRES, J. L. R.; PEREIRA, M. G.; ANDRIOLI, I. Decomposição e liberação de nitrogênio de resíduos culturais de plantas de cobertura em um solo de cerrado. **Revista Brasileira de Ciência do Solo**, Viçosa, v. 29, p. 609-618, 2005.

VAN ELSAS, J. D.; BOERSMA, F. G. H. A review of molecular methods to study the microbiota of soil and the mycosphere. **European Journal of Soil Biology,** Montrouge, v. 47, p. 77-87, 2011.

VAN ELSAS, J. D.; DUARTE, G. F.; KEIJZER-WOLTERS, A. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. **Journal of Microbiological Methods**, Amsterdam, v. 43, n. 2, p. 133-151, 2000**.**

VAN RAIJ, B.; ANDRADE, J. C.; CANTARELLA, H.; QUAGGIO, J. A. **Análise química para avaliação da fertilidade de solos tropicais**. Campinas: Instituto Agronômico, 2001. 285 p.

VAN RAIJ, B.; QUAGGIO, J. A. **Métodos de análise de solo para fins de fertilidade**. Campinas: Instituto Agronômico de Campinas, 1983. 16 p. (IAC. Circular, 63).

VANCE, E. D.; BROOKES, P. C.; JENKINSON, D. S. An extraction method for measuring soil microbial biomass C. **Soil Biology and Biochemistry**, Oxford, v. 19, p. 703-707, 1987.

WALLANDER, H.; NILSSON, L. O.; HAGERBERG, D.; ROSENGREN, U. Direct estimates of C:N ratios of ectomycorrhizal mycelia collected from Norway spruce forest soils. **Soil Biology and Biochemistry**, Oxford, v. 35, n. 7, p. 997-999, 2003.

WILCKE, W.; LILIENFEIN, J. Biogeochemical consequences of the transformation of native Cerrado into *Pinus caribaea* plantations in Brazil. **Plant and Soil**, Dordrecht, v. 238, p. 175-189, 2002.

YU, Y.; LEE, C.; KIM, J.; HWANG, S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. **Biotechnology and Bioengineering**, New York, NY, v. 89, n. 6, p. 670–679, 2005.

ZUMFT, W. G. Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. **Journal of Inorganic Biochemistry**, New York, v. 99, p. 194–215, 2005.

APPENDICES

Appendix A - DNA extraction

Nucleic acid quality and yield are critical factor for a good PCR amplification (SALEH-LAKHA et al., 2011). Using different protocols, different DNA yield and quality were obtained (Table A).

The phenol-CTAB protocol was really effective for the Cerrado samples, but presented several limitations for the soybean soil samples (Table A). Even that the Power Soil kit presented lower yields, we decided to use this kit, since it was more reliable among the different samples.

Table A – Influence of different protocols in DNA yield and quality (n>2)

Extraction	Sample	Depth	DNA ng/ul	280	230	ngDNA/gSolo
Phenol-CTAB	CERRADO B3	0-5	326	1.93	2.13	648.8
	CERRADO B3	20-40	49	2.31	1.82	96.3
	Brachiaria C3	0-5	76	1.85	1.06	188.7
	Brachiaria C3	20-40	15	1.73	0.78	38.5
	Soybean B3	0-5	9	1.4	0.7	21.7
	Soybean B3	20-40	7	1.50	0.65	17.0
SDS/K-acet	CERRADO B3	0-5	42	1.44	1.25	104.5
	CERRADO B3	20-40	27	1.37	1.09	68.5
	Brachiaria C3	0-5	45	1.71	1.63	111.8
	Brachiaria C3	20-40	22	1.16	1.08	55.0
	Soybean B3	0-5	79	1.52	1.43	197.5
	Soybean B3	20-40	33	1.50	1.36	81.3
Power Soil	CERRADO B3	0-5	15	1.02	0.29	56.2
	CERRADO B3	20-40	10	0.75	0.20	40.4
	Brachiaria C3	0-5	14	0.75	0.28	53.5
	Brachiaria C3	20-40	na*	na	na	na
	Soybean B3	0-5	17	0.35	0.15	64.1
	Soybean B3	20-40	na	na	na	na

* na, no available

DNA yield is critical for qPCR studies, especially because the quantity of DNA will be linked to the final result. For example, using different protocols the DNA yield ranged from approximately 15 $ng.\mu l^{-1}$ to approximately 300 $ng.\mu l^{-1}$ (Table A). It means, hypothetically, that using different extraction protocols it can result in an error

of 20 times in the final quantification of a target gene. Additionally, low abundant genes can not be detected if the yield is low (Chapter 7).

DNA quality is also important. For example, the presence of inhibitors may cause several distortions in the qPCR analysis, or even false negatives (Appendix B).

In 2010, a successor of the Power Soil kit was released, the Power Lyzer. Test was performed comparing tropical and temperate soils (Table B). Both kits, Power Soil and Power Lyzer presented better extraction, higher yield and quality, for the temperate soils than for the tropical soils (Table B).

Table B – Comparison of DNA extraction kits for Tropical and Temperate soils (n>2)

Extraction	Soil Sample	DNA ng/ul	280	230
Power Soil	Temperate Forest	24.6	1.75	1.64
	Temperate Garden	37.7	1.83	1.91
	Tropical Forest	11.3	1.69	0.94
	Tropical Agriculture	9.7	1.76	1.20
Power Lyser	Temperate Forest	55.5	1.85	1.86
	Temperate Garden	52.6	1.86	1.86
	Tropical Forest	27.1	1.79	1.86
	Tropical Agriculture	19.2	1.74	1.54

New approaches have been developed to improve DNA extraction and quality, but to the best of our knowledge, up to now no protocol is really reliable for tropical soils. As observed in our tests, yield can be several underestimate; i.e. using the Phenol-CTAB protocol a much higher yield was observed.

Since we could not find an ideal protocol for the tropical samples, we tried to optimize qPCR conditions to increase detection accuracy (Appendix B).

Appendix B – qPCR improvement

We experienced several difficult to adaptation of the qPCR protocols. Amplification using some primers, such as *pmo*A, resulted in good amplification of standards, but not for the samples. Since DNA of the samples was already extracted, we tried re-purification with several protocols, but no one was efficient. Since DNA quality could not be improved, we tried the use of several PCR enhancers (Table C). Additionally, we tested a Taq polymerase resistant to inhibitors (Figure A).

Table C – Tested qPCR enhancers

Reference	Substances	Reason
STEINBERGER et al., 2009	Betaine BSA	It gives AT and GC equal weights Taq stabilization
HENRY et al., 2006	T4 Gene 32 Protein	Taq stabilization
IDT company	DMSO	Open double strain DNA
KlenTaq Company	PEC	Patent protected mix

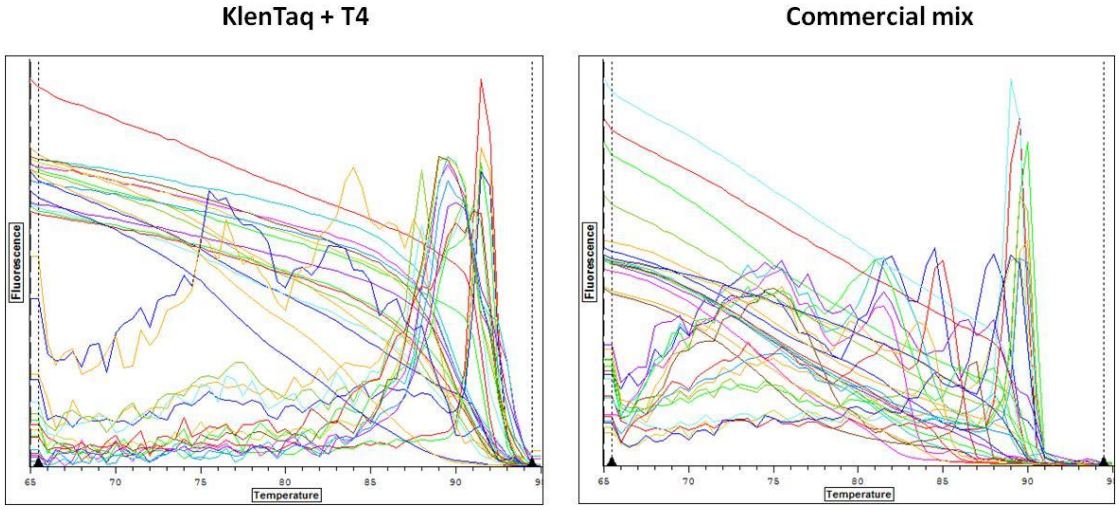

Figure A – Melting curve of qPCR for the gene *pmo*A. In the left the best mix combination (KlenTaq + T4), showing good amplication for samples; primer dimmers were observed only in the negative controls. In the left graphic is the result of the best commercial mix available, showing weak amplification of the samples and high primer dimmer formation

Among all the conditions tested, the best results were obtained with a mix of KlenTaq, T4 Gene 32 Protein and EvaGreen.

Appendix C – Supplementary data

Additional data referent to Chapter 7 is presented bellow (Figures B, C, D, E and Table D). Codes correspond to: Forest (For), soybean (Soy), pasture (Pas); survey day, January 15, 17 or 19; day 16 is Jaguaruna Farm; replicate site B or C.

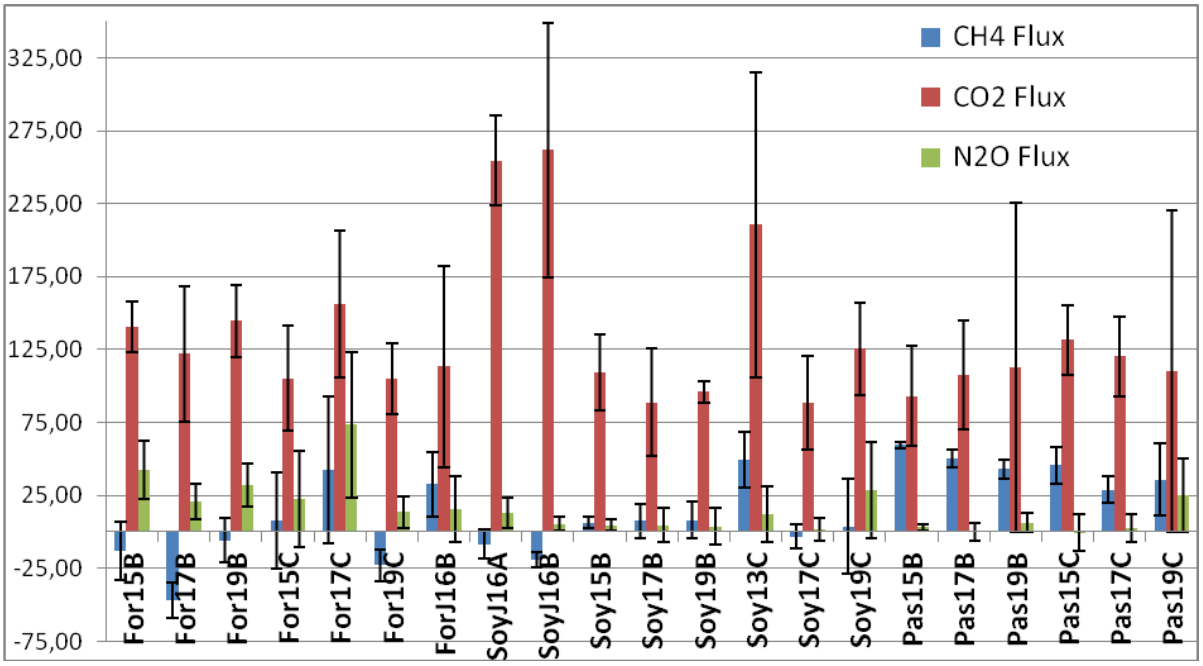

Figure B – GHG measurements presented by survey days

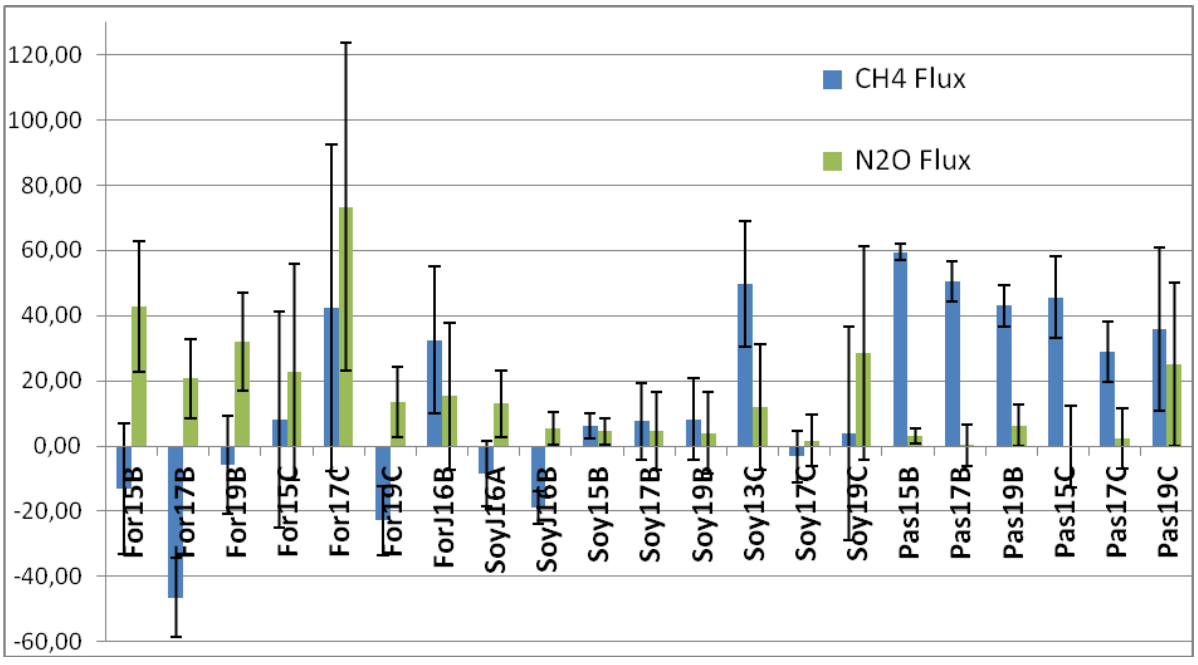

Figure C – Enviromental measurements presented by survey days

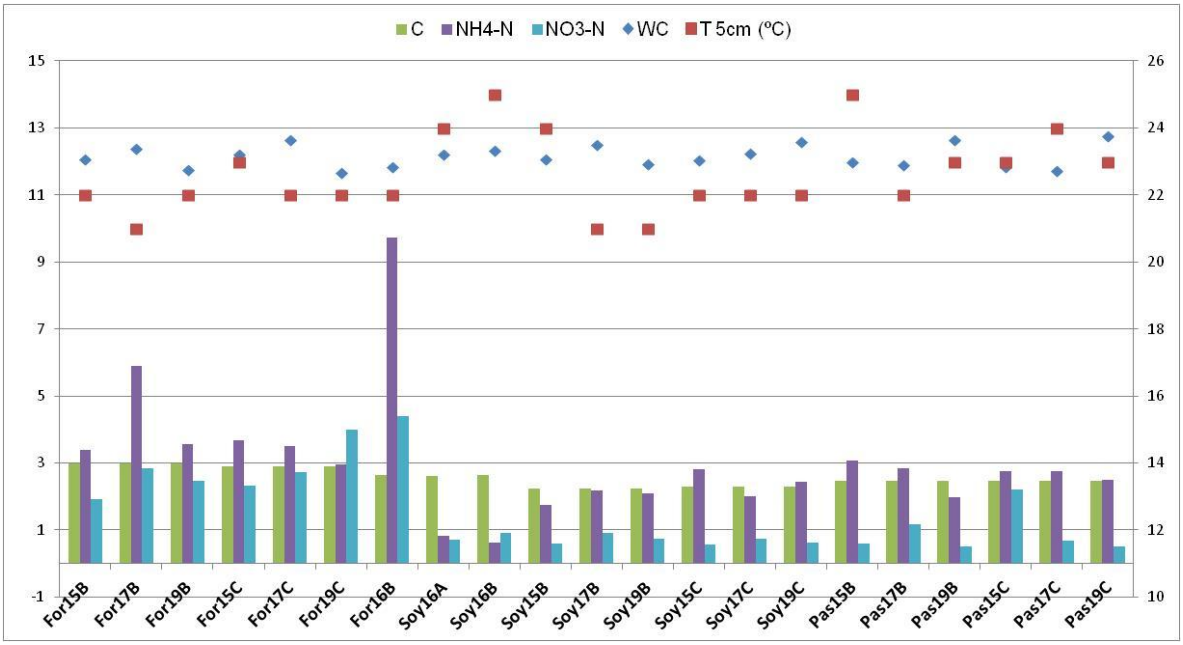

Figure D – Enviromental measurements presented by survey days

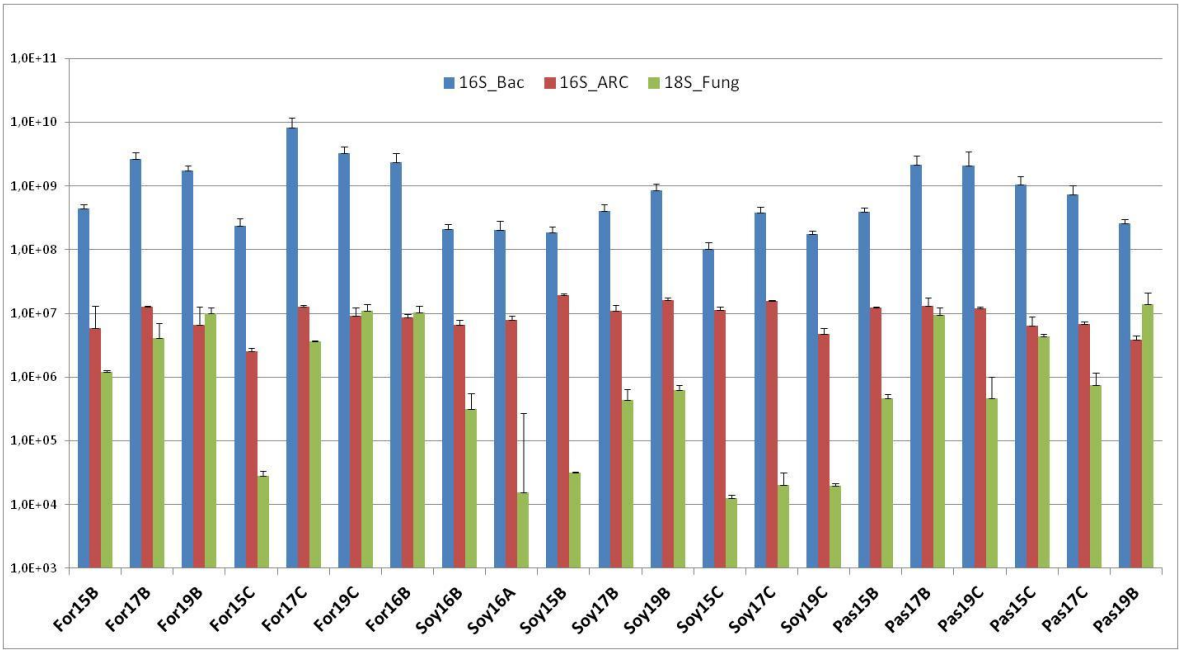

Figure E – Gene quantification presented by survey days

Table D – Gene quantification presented by survey days and replicate plots

Survey	16SBac	16SArc	18SFun	amoA	nirK	nirS	nosZ	pmoA
For15B	4,5E+08	5,8E+06	1,2E+06	2,9E+05	1,7E+06	1,2E+06	1,1E+07	2,6E+07
For17B	2,6E+09	1,3E+07	4,2E+06	7,0E+05	4,6E+06	1,2E+06	1,8E+07	5,8E+07
For19B	1,8E+09	6,5E+06	1,0E+07	4,5E+05	7,2E+06	3,4E+06	2,2E+07	7,9E+07
For15C	2,4E+08	2,5E+06	2,8E+04	3,7E+05	1,3E+06	1,1E+06	5,3E+06	1,2E+07
For17C	8,1E+09	1,3E+07	3,6E+06	5,4E+05	1,4E+07	2,3E+06	4,5E+07	7,7E+07
For19C	3,3E+09	9,1E+06	1,1E+07	8,4E+05	1,1E+07	3,7E+06	3,3E+07	1,1E+08
For16B	2,3E+09	8,6E+06	1,0E+07	9,3E+05	1,1E+07	2,3E+06	2,2E+07	9,1E+07
Soy16B	2,1E+08	6,7E+06	3,1E+05	1,7E+06	6,4E+05	3,8E+05	1,5E+06	9,1E+06
Soy16A	2,0E+08	8,0E+06	1,6E+04	1,4E+06	5,4E+05	5,1E+05	2,0E+06	1,0E+07
Soy15B	1,9E+08	1,9E+07	3,2E+04	7,4E+06	1,1E+06	2,9E+05	1,3E+06	7,3E+06
Soy17B	4,1E+08	1,1E+07	4,4E+05	6,5E+06	2,6E+06	8,6E+05	2,6E+06	1,0E+07
Soy19B	8,6E+08	1,6E+07	6,4E+05	8,3E+06	2,6E+06	1,0E+06	2,7E+06	1,8E+07
Soy15C	1,0E+08	1,1E+07	1,2E+04	4,0E+06	6,0E+05	2,9E+05	1,8E+06	6,6E+06
Soy17C	3,8E+08	1,6E+07	2,0E+04	8,4E+06	4,3E+06	1,3E+06	1,8E+06	1,1E+07
Soy19C	1,7E+08	4,8E+06	2,0E+04	1,6E+06	6,4E+05	8,5E+05	1,1E+06	7,3E+06
Pas15B	3,9E+08	1,2E+07	4,7E+05	3,9E+06	2,5E+06	8,1E+05	8,5E+06	8,9E+06
Pas17B	2,1E+09	1,3E+07	9,4E+06	4,2E+06	5,0E+06	1,3E+07	1,1E+07	7,6E+07
Pas19C	2,1E+09	1,2E+07	4,7E+05	7,1E+06	4,8E+06	6,3E+06	1,3E+07	4,2E+07
Pas15C	1,1E+09	6,4E+06	4,4E+06	1,5E+06	6,1E+06	1,6E+06	1,4E+07	2,9E+07
Pas17C	7,3E+08	6,8E+06	7,4E+05	5,6E+06	3,6E+06	1,3E+06	5,7E+06	2,2E+07
Pas19B	2,6E+08	3,9E+06	1,4E+07	1,5E+06	1,7E+06	4,4E+06	1,5E+06	9,2E+06

* Codes correspond to: Forest (For), soybean (Soy), pasture (Pas); survey day, January 15, 17 or 19; day 16 is Jaguaruna Farm; replicate site B or C.