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Bacterial ecology in Amazonian soils under deforestation and agricultural management

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The first step to getting the things you want out of life is this: Decide what you want.

Ben Stein

RESUMO

NAVARRETE, A.A. **Ecologia bacteriana em solos da Amazônia sob desmatamento e manejo agrícola.** 2012. 136 f. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

Este trabalho de tese avaliou o efeito do desmatamento sobre redes artificiais de associação entre grupos taxonômicos de *Bacteria* e fatores abióticos do solo e redes baseadas em funções bacterianas e fatores abióticos do solo, e utilizou técnicas moleculares e abordagem dependente de cultivo para compreender a dinâmica da comunidade de *Acidobacteria* em solos da floresta Amazônica convertidos em áreas agrícolas para produção de soja. Para estudo das comunidades bacterianas foram utilizadas tecnologias de sequenciamento de nova geração (plataformas 454 GS FLX Titanium da Roche e Illumina HiSeq 2000), PCR quantitativo em tempo-real, método de *fingerprinting* e procedimentos tradicionais para cultivo de bactérias. O objetivo geral desta tese foi alcançado com o desenvolvimento de três diferentes estudos. O *Estudo 1* considerou aproximadamente 425 mil sequências do gene 16S rRNA de *Bacteria* e 266 milhões de sequências de DNA bacteriano obtidas por análise metagenômica a partir de solos coletados em três réplicas verdadeiras de floresta intacta na Amazônia e área desmatada adjacente após corte e queima da cobertura vegetal. Com isso, este estudo mostrou que o desmatamento declina a abundância e altera a estrutura de comunidade de *Verrucomicrobia* no solo e simplifica as redes artificiais de associação entre diferentes grupos bacterianos. A rede artificial de associação entre categorias funcionais e fatores de solo revelou-se mais complexa em solos desmatados, indicando um alto grau de dispersão de risco para a manutenção do funcionamento do solo. Por sua vez, o *Estudo 2* correlacionou a abundância de subgrupos de *Acidobacteria* – com base em aproximadamente 33 mil sequências do gene 16S rRNA de *Acidobacteria* – com fatores abióticos do solo, e mostrou que subgrupos de *Acidobacteria* respondem diferentemente aos efeitos do manejo agrícola de solos da floresta Amazônica dentro de áreas de produção de soja. Este estudo abriu possibilidades de explorar subgrupos de *Acidobacteria* como bio-indicadores dos efeitos do manejo agrícola do solo na região da Amazônia. Por fim, o *Estudo 3* reportou a culturabilidade e detecção molecular de *Acidobacteria* subgrupos 1 e 3 e outros grupos bacterianos presentes em solos Amazônicos em meio de cultura enriquecido com carbono e incubado sob atmosfera hipóxica (2% O_2 [vol/vol], 2% CO_2 [vol/vol] e 96% N_2 [vol/vol]), atestando, assim, a combinação de procedimentos tradicionais de cultivo e técnicas moleculares para a recuperação e detecção de *Acidobacteria* de solos da Amazônia.

Palavras-chave: Ecologia microbiana. Ecologia molecular. Microbiologia do solo. Solos tropicais da Amazônia. Mudanças de uso da terra.

ABSTRACT

NAVARRETE, A.A. **Bacterial ecology in Amazonian soils under deforestation and agricultural management.** 2012. 136 f. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

This thesis assessed effects of Amazonian deforestation on artificial association networks of bacteria to bacteria and to abiotic soil factors and networks based on categories of bacterial functions and abiotic soil factors, and sought a better insight into community of *Acidobacteria* in Amazon soils under agricultural management of soybean based on culture-dependent and molecular approaches. Bacterial community was studied based on next-generation sequencing technologies (Roche GS FLX Titanium and Illumina HiSeq 2000 platforms), quantitative real-time PCR, fingerprinting technique and basic procedures for bacteria culture. The general objective of this thesis was achieved by development of three different studies. The *Study 1* analyzed the total bacterial community based on 16S ribosomal DNA pyrotag (425 thousand sequences), shotgun metagenomics (266 million sequences) and environmental parameters from soil samples collected in three real replicate of intact Amazon rainforest and adjacent deforested site after 2-4 months of forest clearing and burning in the Brazilian Amazon. This study showed that deforestation of Amazon forest soils led to a consistent decline in the abundance of *Verrucomicrobia* and alterations in verrucomicrobial community structure, and simplified association networks among different bacterial taxonomic groups and abiotic soil factors. In order to adapt to this condition function-based associations network were enhanced, indicating a higher degree of risk spreading for the maintenance of soil functioning. The *Study 2*, in turn, correlated relative abundance of *Acidobacteria* subgroups – based on approximately 33 thousand sequences of acidobacterial 16S rRNA genes – and abiotic soil factors, and showed differential response of *Acidobacteria* subgroups to abiotic soil factors in Amazon forest soils into soybean croplands. This study opened the possibilites to explore acidobacterial subgroups as early-warning bio-indicators of agricultural soil management effects in the Amazon area. Lastly, the *Study 3* reported the culturability and molecular detection of *Acidobacteria* subgroups 1 and 3 concomitantly to other bacterial groups from Amazon soils on enriched culture medium with carbon source and incubated for relatively long period in hypoxic atmosphere (2% O_2 [vol/vol], 2% CO_2 [vol/vol] and 96% N_2 [vol/vol]), and validated the combination of traditional procedures for bacteria culture and molecular techniques for recover and detection of *Acidobacteria* from Amazon soils.

Keywords: Microbial ecology. Molecular ecology. Soil microbiology. Amazon tropical soils. Land-use changes.

SUMMARY

1 INTRODUÇÃO

A floresta amazônica desempenha um papel essencial nos processos ecológicos globais, mantendo os maiores corpos de água doce do mundo, controlando a temperatura e precipitação, e balanceando o fluxo de gases atmosféricos. Ela é também um "hot spot" para espécies animais e vegetais da Terra e alvo de décadas de pesquisas em biodiversidade. Ainda assim, a floresta amazônica está na lista dos escossistemas menos compreendidos no que tange à diversidade microbiana. Embora alguns trabalhos recentes tenham buscado caracterizar as comunidades microbianas em solos de diferentes regiões da Amazônia, a ecologia de micro-organismos é especialmente pouco estudada nestes solos, tornando imperativa a necessidade de melhor compreendê-la frente à notória influência antrópica na complexidade, heterogeneidade, dinâmica e interação dos diversos fatores físicos, químicos e biológicos do sistema edáfico. Alterações ao nível dos ecossistemas da Amazônia prevêem efeitos significativos sobre as emissões globais de gases do efeito estufa, aquecimento global, recursos hídricos e degradação do solo. Todos esses efeitos decorrem da intensificação do uso da terra, que pode trazer desvantagens ambientais, principalmente no ambiente do solo, tais como perda de matéria orgânica, desestruturação e posterior erosão, esgotamento da fertilidade e poluição com fertilizantes e pesticidas (CERRI et al., 2005). Diante do atual contexto, deve-se procurar maneiras inovadoras e criativas para manter os serviços dos ecossistemas amazônicos mediante a preservação da biodiversidade.

Por longo tempo o impacto antrópico na área da Amazônia foi mantido a um ritmo mínimo. A partir da década de 1960, políticas de incentivo do governo brasileiro desencadearam a exploração madeireira e pecuária elevando o desmatamento a um ritmo médio de 20.000 quilômetros quadrados por ano. A população humana na região da Amazônia brasileira aumentou de 6 milhões em 1960 para 25 milhões em 2010, e a cobertura florestal nesta região tem declinado para 80% da sua área original (INPA, 2011). Uma crescente demanda por grãos, fibras e biocombustíveis tem criado uma nova fronteira agrícola de 1 milhão de quilômetros quadrados. Com a alta probabilidade de abertura de novas áreas (ASNER et al., 2006) e a susceptibilidade resultante de seca e incêndios, não apenas o equilíbrio natural como também a produtividade da floresta encontra-se ameaçada. Além disso, se o desmatamento for mantido sob o ritmo atual, as perdas de biodiversidade são estimadas em 22% do total das espécies de plantas do planeta (KREFT; JETZ, 2007). No

entando, até o momento não há dados suficientes para estimar os impactos do desmatamento nas comunidades microbianas associadas à floresta amazônica.

Dada a importância ecológica da Amazônia e as ameaças do desenvolvimento que a encerram, é surpreendente que microbiologistas e ecólogos microbianos tenham devotado tão pouca atenção a esta região. Bornemann e Triplett (1997) relataram o primeiro estudo independente de cultivo tratando da diversidade microbiana em solos da Amazônia, onde foram amostradas 98 sequências do gene 16S rRNA de dois diferentes solos no estado do Pará e concluído que todas as sequências eram únicas. Quando Schloss e Handelsman (2005) utilizaram o conjunto de dados de Bornemann e Triplett (1997) para estimar a riqueza de unidades taxonômicas operacionais, determinaram somente dois pares de sequências idênticas, confirmando, assim, a extensiva diversidade bacteriana em solos da Amazônia. Fierer e Jackson (2006) compararam comunidades bacterianas em 98 amostras de solo coletadas em um transecto estabelecido da América do Norte à América do Sul, e mostraram que os solos da Amazônia peruana, com menores valores de pH, foram também os que apresentaram menor riqueza de filotipos de bactérias. Kim et al. (2007) relataram resultados que foram intermediários aos obtidos pelos estudos previamente citados. Estes autores analisaram a diversidade bacteriana em um solo de floresta primária da Amazônia Ocidental, com base em bibliotecas de 50 clones do gene 16S rRNA, e identificaram *Acidobacteria* como um *taxon* predominante, além de estimar que as comunidades bacterianas nestes solos foram moderadas em riqueza taxonômica. Jesus et al. (2009) e Navarrete et al. (2010) mostraram que mudanças de uso da terra alteram a estrutura de comunidades microbianas presentes em solos da Amazônia e também encontraram *Acidobacteria* dentre os *taxa* mais abundantes no solo.

Acidobacteria está entre os grupos bacterianos mais comuns, e com elevada abundância de sequências do gene 16S rRNA no ambiente do solo. Apesar da elevada abundância de sequências do gene 16S rRNA de *Acidobacteria* em diferentes ambientes, incluindo o nicho da rizosfera (CHOW et al., 2002; KUSKE et al., 2002; GREMION et al., 2003; QUAISER et al., 2003; FIERER et al., 2005; STAFFORD et al., 2005; JANSSEN, 2006; SANGUIN et al., 2006; De CÁRCER et al., 2007; SINGH et al., 2007; KIELAK et al., 2009; DeANGELIS et al., 2009; JONES et al., 2009; NAETHER et al., 2012), a ecologia deste grupo bacteriano é pouco compreendida, o que pode ser atribuído principalmente aos poucos membros cultivados e depositados em coleções de cultura, sem nenhum representante

isolado de solos tropicais da Amazônia (KISHIMOTO et al., 1991; LIESACK et al., 1994; COATES et al., 1999; BRYANT et al., 2007; EICHORST et al., 2007; FUKUNAGA et al., 2008; KOCH et al., 2008; LEE et al., 2008; NUNES da ROCHA et al., 2009; WARD et al., 2009; KULICHEVSKAYA et al., 2010; PANKRATOV; DEDYSH, 2010; EICHORST et al., 2011; MÄNNISTÖ et al., 2011; PANKRATOV et al., 2011).

Outros estudos conduzidos na área da Amazônia brasileira também têm cooperado com a avaliação do impacto de mudanças de uso da terra sobre as comunidades microbianas presentes no solo (CENCIANI et al., 2009; O'NEILL et al., 2009; GROSSMAN et al., 2010; PAZINATO et al., 2010; TAKETANI; TSAI, 2010; NAVARRETE et al., 2011; GERMANO et al., 2012; GUIMARÃES et al., 2012; SILVA et al., 2012; NAVARRETE et al., 2012). No entanto, apesar deste aumento recente da apreciação acerca das comunidades microbianas em solos da região Amazônica, relativamente pouco é conhecido sobre as associações entre grupos taxonômicos bacterianos nestes solos e como as funções desempenhadas por grupos bacterianos são afetadas por atividades antrópicas, tais como o desmatamento e o manejo agrícola do solo. Estudos visando identificação de grupos microbianos capazes de atuar como indicadores sensíveis de efeitos de práticas de manejo agrícola do solo também são de necessidade atual para a sustentabilidade dos ecossistemas Amazônicos.

Assim, tendo como base questões científicas formuladas no âmbito dos novos desafios acerca da compreensão das comunidades microbianas em solos da Amazônia, esta tese foi definida e composta por três diferentes estudos. Os *Estudos 1* e *2* foram desenvolvidos a partir de abordagens independentes de cultivo; e um terceiro estudo (*Estudo 3*) integrou método tradicional de cultivo e técnicas moleculares. O *Estudo 1* buscou compreender como o desmatamento na região da Amazônia afeta a abundância de grupos taxonômicos bacterianos presentes no solo e as redes artificiais de associações entre os diferentes grupos taxonômicos e suas relações funcionais com fatores abióticos do solo. O *Estudo 2* fez uma estimativa acurada da abundância do filo *Acidobacteria* em solos da floresta Amazônica convertidos em áreas de produção de soja no sudeste da Amazônia brasileira, com a finalidade de obter esclarecimento suficiente acerca da dinâmica da comunidade de *Acidobacteria* (ao nível taxonômico de classe – subgrupos) em função de alterações nos fatores abióticos do solo. Por fim, o *Estudo 3* validou tratamentos testados em Stevenson et al. (2004) e Davis et al. (2005), tais como estratégia de

cultivo, detecção molecular de colônias bacterianas filo-específicas e condição atmosférica de incubação, com o propósito de atestá-los para a recuperação e detecção em meio de cultura de representantes do filo *Acidobacteria* de solos da Amazônia brasileira.

1.1 Hipóteses

O *Estudo 1* apresentado nesta tese buscou testar a hipótese de que a estrutura das comunidades bacterianas em solos da Amazônia responde aos efeitos do desmatamento, alterando as associações entre os diferentes grupos taxonômicos e suas relações funcionais com fatores abióticos do solo.

Por sua vez, os *Estudos 2* e *3* aqui descritos focaram sobre a comunidade de *Acidobacteria* de solos da Amazônia e buscaram testar as seguintes hipóteses:

(Estudo 2) A estrutura e abundância da comunidade de *Acidobacteria* respondem ao manejo agrícola de solos da floresta amazônica convertidos em áreas de produção de soja.

(Estudo 3) Representantes do filo *Acidobacteria* podem ser recuperados concomitantemente com outros grupos bacterianos de solos da Amazônia utilizando meio de cultura enriquecido com carbono e incubado sob atmosfera hipóxica por período de tempo relativamente longo.

1.2 Objetivos

1.2.1 Objetivo geral

O objetivo geral desta tese foi avaliar o efeito do desmatamento de floresta intacta da Amazônia sobre a estrutura da comunidade bacteriana e redes de associações bacterianas no ambiente do solo e a resposta da comunidade de *Acidobacteria* ao manejo agrícola de solos da floresta amazônica convertidos em áreas agrícolas para a produção de soja. Constituiu ainda objetivo desta tese a validação de procedimentos de cultivo previamente publicados visando atestá-los para a recuperação e detecção na superfície de meio de cultura de representantes do filo *Acidobacteria* de solos tropicais da Amazônia brasileira.

Para alcançar o objetivo principal desta tese foram utilizadas tecnologias de sequenciamento de nova geração, tais como as plataformas *454 GS FLX Titanium* da Roche e a *Illumina HiSeq 2000*, e método quantitativo como o PCR quantitativo em tempo real e de *fingerprinting* (T-RFLP - *Terminal Restriction Fragment Length Polymorphism*) em associação com métodos estatísticos e de bioinformática. Método de cultivo tradicional foi combinado com técnicas moleculares em um dos estudos aqui descritos.

1.2.2 Objetivos específicos

Para alcançar o objetivo geral desta tese, os seguintes objetivos específicos foram considerados:

- Estimar a abundância de grupos taxonômicos bacterianos e realizar análise metagenômica em amostras de solo coletadas em três réplicas verdadeiras de floresta intacta da Amazônia e área desmatada adjacente logo após o corte e queima da cobertura vegetal no sudeste da Amazônia brasileira, e avaliar o efeito do desmatamento sobre os diferentes grupos taxonômicos bacterianos e redes artificiais de associação bactéria-bactéria-solo e função-função-solo.

- Estimar a abundância de subgrupos de *Acidobacteria* presentes em solo sob menor influência das raízes em duas áreas agrícolas sob cultivo de soja e floresta intacta adjacente no sudeste da Amazônia brasileira, e na rizosfera de soja amostrada em mesocosmos mantidos em casa-de-vegetação, e correlacioná-la com fatores abióticos do solo.

- Testar e validar procedimentos de cultivo e detecção de representantes do filo *Acidobacteria* para solos tropicais da Amazônia brasileira baseado em meio de cultura enriquecido com carbono e período de tempo relativamente longo de incubação sob atmosféra hipóxica.

1.3 Estrutura e organização da tese

Esta tese está estruturada a partir da proposta de apresentação de elementos pré-textuais, textuais – um texto inicial introdutório, seguido de três estudos com apresentação em formato de artigo científico redigidos em língua inglesa – e pós-textuais. Todos os elementos atendem a condição de conteúdos integrados que podem ser examinados e consultados em um único documento.

2 INTRODUCTION

The Amazon forest plays an essential role in global ecological processes, maintaining the largest fresh water bodies of the world, controlling temperature and precipitation, and balancing the flux of atmospheric gases. It is also the largest biological reservoir of animal and plant species on Earth and the target of decades of biodiversity research. Yet it is among the least understood of the Earth's ecosystems regarding microbial diversity. Although recent studies have sought characterize the microbial communities in soils from different Amazon regions, aspects in microbial ecology are especially few studied in these soils, making imperative understand them due the antropic influence in complexity, heterogeneity, dynamic and interaction of physical, chemical and biological factors of the edaphic system. This shift at ecosystem is predicted to have significant effects on global emissions of greenhouse gases, global warming, soil degradation, and water resources. Many effects occur in consequence of intensification in land use that can characterize environmental disadvantages, mainly in soil, such as losses of organic matter, disruption and subsequent erosion, fertility depletion and pollution with fertilizers and pesticides (CERRI et al., 2005). Thus, it is necessary look for innovative and creative ways to mantain the Amazonian ecosystem services by preserving biodiversity.

Until the 1960's, human impact in Amazon area was kept to a minium, but once the Brazilian Government encouraged logging and cattle ranching, deforestation started at an average of 20,000 Km^2 a year. The human population of the Brazilian Amazon region has increased from 6 million in 1960 to 25 million in 2010, and the forest cover for this region has declined to about 80% of its original area (INPA, 2011). An increasing demand for grains, fibers, and biofuel is now creating a new agricultural frontier of 1 million Km^2. With the ongoing high probability of clearing after logging (ASNER et al., 2006), and the resulting susceptibility to drought and fires, not only are the long-term health and productivity of the forest threatened. In addition, the biological losses due to deforestation are estimated to be approximately 22% of the total plant species of the planet, if deforestation continues at the same rate as today (KREFT; JETZ, 2007). Yet, to this date we do not even have baseline data to estimate the impacts of Amazonian deforestation on bacterial community.

Given the ecological importance of the Amazon region and the threats to its survival, it is surprising that microbiologists and microbial ecologists have largely ignored this region. Bornemann and Tripplett (1997) reported the first culture-independent study of soil microbial

diversity in the Amazon area. They sampled 98 bacterial 16S rRNA gene sequences from two different soil samples in the state of Pará and concluded that all sequences were unique. When Schloss and Handelsman (2005) applied DOTUR, a computer program to estimate species richness, to this data set, they found only two pairs of identical sequences, confirming this extensive bacterial diversity in Amazon soils. Fierer and Jackson (2006) compared bacterial communities in 98 soil samples across North and South America, and showed that a Peruvian Amazon soil displaying the lowest pH was also the least diverse. Kim et al. (2007) reported results that were intermediate to the previous studies. These authors surveyed bacterial diversity in a pristine soil from the Western Amazon forest using clone libraries of 50 16S rRNA gene sequences, and identified *Acidobacteria* as the predominant taxon. They also estimated that the bacterial communities in these soils were of moderate taxonomic richness. Jesus et al. (2009) and Navarrete et al. (2010) showed that land use changes alter microbial community structure in Amazon soils, and identified *Acidobacteria* among predominant bacterial taxa in soil.

Acidobacteria are ubiquitous and among the most abundant bacterial phyla in soils by 16S rRNA gene-based molecular surveys. In spite of their high abundance, including soil and rhizosphere niches (CHOW et al., 2002; KUSKE et al., 2002; GREMION et al., 2003; QUAISER et al., 2003; FIERER et al., 2005; STAFFORD et al., 2005; JANSSEN, 2006; SANGUIN et al., 2006; De CÁRCER et al., 2007; SINGH et al., 2007; KIELAK et al., 2009; DeANGELIS et al., 2009; JONES et al., 2009; NAETHER et al., 2012), little information is available on their ecology, which is mainly due to the lack of culturable representatives in bacterial collections, without isolated representatives from Amazon tropical soils (KISHIMOTO et al., 1991; LIESACK et al., 1994; COATES et al., 1999; BRYANT et al., 2007; EICHORST et al., 2007; FUKUNAGA et al., 2008; KOCH et al., 2008; LEE et al., 2008; NUNES da ROCHA et al., 2009; WARD et al., 2009; KULICHEVSKAYA et al., 2010; PANKRATOV; DEDYSH, 2010; EICHORST et al., 2011; MÄNNISTÖ et al., 2011; PANKRATOV et al., 2011).

Other studies in the Brazilian Amazon have also contributed with the assessement of land use changes impacts on soil microbial communities (CENCIANI et al., 2009; O'NEILL et al., 2009; GROSSMAN et al., 2010; PAZINATO et al., 2010; TAKETANI; TSAI 2010; NAVARRETE et al., 2011; GERMANO et al., 2012; GUIMARÃES et al., 2012; SILVA et al., 2012; NAVARRETE et al., 2012). However, despite the increased appreciation of

belowground microbial communities in the Amazonian region, little is still known about impacts of deforestation and agricultural management on microbial functions and associations among bacterial taxa in these soils. Studies focusing on soil bio-indicators for agricultural management effects are imperative for the sustentability of Amazonian ecosystems.

Thus, this thesis was defined based on scientific questions formulated at the light of new challenges encompassing microbial communities in Amazon soils, and it is composed by three different studies. The *Studies 1* and *2* were developed based on culture-independent approaches; and a third study (*Study 3*) combined traditional procedures for bacteria culture and molecular techniques. The *Study 1* sought understand the effects of Amazonian deforestation on bacterial taxonomic groups inhabiting the soil, taxonomy-based networks and function-based networks of bacteria and abiotic soil factors. The *Study 2* made an accurate estimate of acidobacterial abundance in bulk soil from soybean croplands and adjacent native forests, and mesocosm soil from soybean rhizosphere in order to obtain a better insight into the ecological characteristics of *Acidobacteria* (at taxonomic level of class – subgroups) in Amazon soils and in soils recently converted into cultivation. In addition, the *Study 3* tested and validated proceedings published by Stevenson et al. (2004) and Davis et al. (2005), such as strategies of cultivation and molecular detection of target bacterial colonies, in order to indicate a procedure for recover and detection on culture medium of acidobacterial representatives from Brazilian Amazon soils.

2.1 Hypotheses

The *Study 1* presented in this thesis sought to test the hypothesis that bacterial community structure in Amazon soils responds to deforestation effects altering the associations among different bacterial taxonomic groups and their correlations with abiotic soil factors.

In turn, the *Studies 2* and *3* described in this thesis focused on acidobacterial community in Amazon soils in order to test the following hypotheses:

(Study 2) Acidobacterial community structure and abundance respond to agricultural management of Amazon forest soils into soybean croplands.

(Study 3) Acidobacterial representatives can be recovered concomitantly to other bacterial groups from Amazon soils on enriched culture medium with carbon source and relatively long period of incubation in hypoxic atmosphere.

2.2 Objectives

2.2.1 General objectives

The general objetive of this thesis was to assess the effects of Amazonian deforestation on bacterial community structure and association networks of bacteria in soil and get a better insight about the response of acidobacterial community to agricultural management of Amazon soils into soybean croplands. In addition, this work of thesis sought validate procedures described in the literature in order to certify them for recovering and detection on culture media of acidobacterial representatives from Brazilian Amazon soils.

To achieve the main objective of this thesis were used next-generation sequencing technologies, such as Roche 454 GS FLX Titanium and Illumina HiSeq 2000 sequencing platforms. Quantitative methodologies as real-time quantitative PCR and fingerprinting techniques (T-RFLP – Terminal Restriction Fragment Length Polymorphism) were also used in combination with statistical and bioinformatics tools. Traditional cultivation method was combined with molecular techniques in a study here described.

2.2.2 Specifics objectives

To achieve the general objective of this thesis the following specific objectives were considered:

- To estimate the abundance of bacterial taxonomic groups and develop metagenomic analysis based on soil samples taken in three replicate sites of mature forest and adjacent deforested site after 2-4 months of forest clearing and burning in the Brazilian Amazon, and assess the effects of deforestation on different taxonomic groups of bacteria and artificial association networks bacteria-bacteria-soil and function-function-soil.

- To estimate the abundance of *Acidobacteria* subgroups present in bulk soil samples from soybean croplands and adjacent native forests, and mesocosm soil samples from soybean rhizosphere, and correlate it with abiotic soil factors.
- To test and validate procedures of cultivation and detection for acidobacterial representatives from Amazon tropical soils based on enriched culture medium with carbon source and relatively long period of incubation in hypoxic atmosphere.

2.3 Structure and organization of the thesis

This thesis describes pre-textual, textual – an introductory initial text followed by three studies presented in scientific manuscript format written in English language – and post-text elements. All elements meet the condition of integrated content that can be viewed and examined in a single document.

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3 AMAZONIAN DEFORESTATION ALTERS BACTERIAL NETWORKS AND VERRUCOMICROBIAL COMMUNITIES IN SOIL

ABSTRACT

Deforestation in tropical regions is known to have large effects on biodiversity, ecosystem functioning and soil fertility. However, little is known about the impacts of tropical deforestation on the soil-borne microbial communities that drive soil functioning. Here, we analyzed soil bacterial community from three real replications of mature forest and adjacent deforested site after 2-4 months of forest clearing and burning in the Brazilian Amazon using network analysis approach based on 16S ribosomal DNA pyrotag, shotgun metagenomics and measurements of environmental parameters to address the question whether or not the complexity of the association networks bacteria-bacteria-soil and function-function-soil is affected by slash-and-burning of intact mature Amazon forest. In addition, we also focused more specifically on one of the most shifted bacterial phyla, the *Verrucomicrobia*, examining the community size and structure by quantitative real-time PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP) fingerprinting, respectively. As a response to deforestation we found a less complex taxonomy-based network in deforested soils as compared to forest soils. However, the function-based network was more complex in deforested soils as compared to forest soils. The relative proportion of *Verrucomicrobia* declined in response to deforestation (from approximately 5% to 2.5%), and principal component analysis of T-RFLP revealed disparate verrucomicrobial community structures in both areas. We conclude that deforestation of Amazon forest soils quickly leads to simplification of association networks among different bacterial taxonomic groups and abiotic soil factors and declines *Verrucomicrobia*. In order to adapt to this condition function-based associations network were enhanced, indicating a higher degree of risk spreading for the maintenance of soil functioning.

Keywords: slash-and-burning; land-use changes; tropical rainforest; soil microbial ecology; ecosystem functioning; high throughput DNA sequencing

3.1 Introduction

Humans have inhabited the Amazon basin for many thousands of years. However, the expansion and intensification of agriculture, logging and urban footprints during the past few decades are changing the landscape at an unprecedented rate (DAVIDSON et al., 2012). The human population of the Brazilian Amazon region has increased from 6 million in 1960 to 25 million in 2010, and the forest cover for this region has declined to about 80% of its original area (INPA, 2011). Land use change is one of the greatest threats to biodiversity worldwide, and one of the most devastating land use changes, especially in the tropics, is the destruction of intact forests (FUJISAKA et al., 1998).

The Amazon rain forest is a vast, yet vulnerable, hotspot of biodiversity, and the maintenance of soil fertility is critical to sustaining these diverse ecosystems. Thus, increased attention has recently been paid to belowground biodiversity in the Amazonian region, and a number of studies have sought to interrogate the microbial communities resident to Amazonian soils and to assess the impact of forest destruction and recovery (BORNEMAN; TRIPLETT, 1997; KIM et al., 2007; CENCIANI et al., 2009; JESUS et al., 2009; O'NEILL et al., 2009; NAVARRETE et al., 2010, 2011, 2012; PAZINATO et al., 2010; TAKETANI; TSAI, 2010). However, despite the increased appreciation of belowground microbial diversity in the Amazonian region, little is still known about impacts on microbial functions by deforestation and about the associations among bacterial taxa in these soils, which are recognized to be strong indicators of changes in soil ecosystems (BARBERÁN et al., 2011).

Advances in next-generation DNA sequencing methods, such as pyrosequencing (MARGULIES et al., 2005) and shotgun metagenome (VENTER et al., 2004), have made sequence analyses sufficiently affordable to allow for detailed examination of soil-borne microbial community structure (ROESCH et al., 2007; LIU et al., 2007; SOGIN et al., 2006) and function (FIERER et al., 2012). Such approaches not only facilitate the assessment of microbial distribution patterns at various taxonomic levels, but also permit the exploration of co-occurrence patterns of soil microorganisms and responses to key environmental gradients, such as moisture, pH, vegetation and biogeochemical cycles (PROULX et al., 2005; FIERER et al., 2008; LAUBER et al., 2009; BARBERÁN et al. 2011; ANDREOTE et al., 2012).

In the present study, we used a pyrosequencing approach, directed at the V4 16S rRNA gene region to examine soil bacterial community structure, and shotgun metagenomic

analysis based on ultra-deep soil DNA sequencing for function prediction across three real replication of mature forest and adjacent deforested site in the Brazilian Amazon. We further analyzed patterns of bacterial co-occurrence and bacterial distributions with respect to key environmental factors to examine the impact of deforestation on association networks bacteria-bacteria-soil (BBS) and function-function-soil (FFS), with the following questions in mind: Which bacterial taxonomic groups are responsive to deforestation effects in Amazon forest soils? Are BBS and FFS networks more complex in Amazon forest soils than in deforested soils? Given the responsiveness of *Verrucomicrobia* to deforestation that we observed in this study, and the importance of this poorly understood phylum (BERGMANN et al., 2011), we also carried out a series of experiments to track the dynamics of this phylum. For this purpose, the abundance of *Verrucomicrobia* was determined by quantitative real-time PCR, and verrucomicrobial community structure was assessed by terminal restriction fragment polymorphism (T-RFLP), to examine if the *Verrucomicrobia* might represent a useful model group for tracking the effects of deforestation on soil-borne microbial communities.

3.2 Materials and methods

3.2.1 Site description and soil sampling

Soil samples were collected in three areas of an approximate region of 60 ha in the Southeastern Brazilian Amazon, State of Mato Grosso, Brazil: Area 1 (15°11'45'' S and 54°03'31'' W), Area 2 (14°21'38'' S and 54°21'27'' W) and Area 3 (13°21'57'' S and 54°54'24'' W) (Supplementary Table 1). Oxisoil is the predominant soil order at these field sites (SEPLAN, 2001). The climate in the region is classified as Am (Koppen's classification), with annual average temperature of 28°C and average precipitation of 2000 mm. The rate of deforestation in the Southeastern Brazilian Amazon is high and mainly due to intensive mechanized agriculture into areas previously covered by intact forest (MORTON et al., 2006). The three sampling areas were considered real replications and identified just after forest clearing. Soil samples were taken from deforested highland sites 2-4 months after forest clearing and burning (Supplementary Table 1). At the same time, soil samples were also collected from adjacent native forests. At each site, the soil samples were collected from five

sampling points (points randomly named in field by A, B, C, D and E). One central sampling point and other four sampling points (at least 50 m apart from the central point) directed towards the north, south, east and west of the central point. Soil samples were taken from the 0 to 20 cm topsoil layer. First, the litter layer was removed, and then the soil samples were taken using a 5 cm (diameter) aseptic cylindrical core. A total of 30 soil samples were collected (3 sampling areas x 2 sampling sites per area x 5 sampling points per site). Samples were transported to the laboratory under ice, kept separately and stored at −20°C until processing within 72 h after sampling.

3.2.2 Abiotic soil factors

For soil physicochemical analysis, soil sample consisted of a composite sample for each sampling site by mixing five sub-samples collected from the sampling points. From each composite sample, a sub-sample was removed, air dried and passed through a 2 mm mesh sieve, and then analyzed according to EMBRAPA (1997) at the Department of Soil Science at the University of São Paulo (ESALQ-USP) for the soil fertility properties. Soil pH was measured in a 1:2.5 soil:water suspension. Exchangeable Al, Ca and Mg were extracted with KCl 1 mol L^{-1}. Calcium and Mg were determined by atomic absorption spectrometry and Al by acid-base titration. Phosphorous and K were extracted by ion exchange resin. Potential acidity (H + Al) was estimated by an equation based on the pH determined in SMP buffer solution (pH SMP). Available micronutrients (Fe, Mn, Zn, and Cu) were extracted by Mehlich 1 and determined by atomic absorption spectrometry. Boron was extracted with hot water and determined by spectrophotometry with azomethine-H at 420 nm. Some of the results allowed the calculation of other parameters-such as: exchangeable bases (SB), the sum of Ca, Mg and K; cation exchange capacity (CEC), the sum of Ca, Mg, K, Al, and H; base saturation (V), the percentual relation of SB and CEC; and Al saturation (m%), the percentual relation of exchangeable Al and CEC. Soil texture was determined with Bouyoucos densimeter after shaken the soil vigorously with NaOH 1 mol L^{-1} as dispersant. Soil moisture content (H) was determined by the gravimetric method.

3.2.2.1 Statistical analysis for soil factors

Analyses of similarity (ANOSIM) of soil physicochemical properties were performed considering the differences in forest and deforested soils. A distance matrix (Euclidean metric) was constructed with non-transformed data. ANOSIM was carried out using Primer six (version 6.1.5, Primer-E Ltd., Plymouth, UK).

3.2.3 Isolation of DNA from soil, amplification and 16S rRNA gene pyrosequencing

All environmental DNA extractions were performed with 250 mg of soil (wet weight) using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. DNA extraction was performed in duplicate for each of 30 soil samples and stored at −20°C until use. Amplicons for barcoded pyrosequencing were generated by PCR using the primer set 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (LAUBER et al., 2009) targeting the V4 region of the bacterial 16S rRNA gene. The 515F primer included the Roche 454-B pyrosequencing adapter and GT linker, while 806R included the Roche 454-A sequencing adapter and GG linker. A 12-bp barcode (unique to each sample) was included in both primers. Soil DNA from three sampling points per site (points A, C and E from a total of five sampling points randomly named in field by A, B, C, D and E) was used as template in PCR reactions, which reactions were performed in duplicate for each soil DNA sample. The reactions contained 2.5 × reaction buffer, 0.2 mM of each dNTP, 0.5 μM of each primer (Alpha DNA, Montreal, Canada), 10 ng of template DNA, 0.056 U of FastStart High-Fidelity PCR System enzyme blend (Roche Applied Sciences, Indianapolis, IN, USA) and sterile water to 25 μL final volume. After optimization, the following thermocycling conditions were chosen: initial denaturation for 5 min at 95°C; 25 cycles of 30 s at 95°C, 1 min at 53°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. After product integrity confirmation by agarose gel electrophoresis, amplicons from 10 reactions were combined and cleaned with the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). PicoGreen-base quantification permitted the normalization for construction of multiplex amplicon pool (HARRIS et al., 2010). The samples were sequenced on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA) (Macrogen Inc. Company, South Korea).

3.2.4 Pyrosequencing data preprocessing and statistical analysis

DNA sequences and quality information were extracted from the Standard Flowgram Format (SFF) files using the SFF converter tool in the Galaxy interface (GOECKS et al., 2010). Sequences were further analyzed with the Qiime scripts, version 1.2.1 (CAPORASO et al. 2010), which were made available in the Galaxy interface. Firstly, all the sequences that had a perfect match to the primer sequences, contained no homopolymer run exceeding six nucleotides and showed no ambiguous characters were assigned to samples by matching to barcode sequences. Reverse complementary sequences were put in the forward orientation and assigned to samples by partially matching to barcode sequences. This step was confirmed by the V-REVCOMP tool using Hidden Markov Models (HMMs) (HARTMANN et al., 2011). Secondly, sequence errors introduced during pyrosequencing were detected and removed using the option for titanium data in the Denoiser 0.91 program (Reeder and Knight, 2010). The sequences were also checked for PCR chimeras using UCHIME version 4.2.40 (EDGAR et al., 2011). Only sequences with a length between 200 and 350 base-pairs were accepted and bases with a quality score lower than 20 were trimmed. The low quality sequences were discarded. The obtained high quality sequences were clustered into Operational Taxonomic Units (OTUs) using UCLUST version 1.2.21 (EDGAR, 2010) with a minimum sequence identity cutoff of 97%. For each OTU, the most abundant sequence was selected as a representative for all sequences within the given OTU. Taxonomy was assigned to the representative sequences using the Ribosomal Database Project (RDP) 2 classifier (release 10.4), with a minimum support threshold of 60%. Finally, the OTU table was filtered for specific taxonomic terms using scripts provided in Qiime. The relative abundance of different bacterial groups was estimated in each bacterial community by comparing the number of sequences classified as belonging to the specific bacterial groups versus the number of classified bacterial sequences per sample. One-way ANOVA was used to determine the significance of the differences between forest and deforested soils. The comparison of soils was based on *post hoc* analysis using Tukey's HSD test. Explicit relationships between relative abundances of bacterial groups and soils from forest or deforested sites were examined by constrained ordination generated by redundancy analysis (RDA) in CANOCO 4.5 (ter BRAAK; ŠMILAUER, 2002).

3.2.5 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) targeting the 16S rRNA gene was performed to assess the size of the verrucomicrobial and total bacterial communities in each of 30 soil samples. As standard, amplicons of *Verrucomicrobia spinosum* (DSMZ 4136) and bacterial clone from an environmental sample were obtained by PCR using primers pA-F (5'-AGAGTTTGATCCTGGCTCAG-3') (EDWARDS et al., 1989) and 1378R (5'-CGGTGTGTACAAGGCCCGGGAAGG-3') (HEUER et al., 1997), purified (QIA-quick PCR purification kit, Qiagen, Venlo, the Netherlands) and ligated into the pGEM-T vector (Promega, Leiden, the Netherlands). Ligation products were transformed with *E.coli* JM109 competent cells (Promega). Cloned inserts were re-amplified using primers SP6 and T7, and plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Venlo, the Netherlands) from appropriate clones (i.e. belonging to the desired target group). DNA standard curves were generated by producing a dilution series of 10^4 to 10^8 copies μL^{-1} using duplicate 10-fold dilutions of isolated plasmid DNA. For qPCR of 16S rRNA gene fragments from *Verrucomicrobia* and from total bacteria, we used the following primer pairs: Ver53 (5'-TGGCGGCGTGGWTAAGA-3') (STEVENSON et al., 2004) Eub518 (5'-ATTACCGCGGCTGCTGG-3') (MUYZER et al., 1993) and Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') (LANE, 1991)/Eub518 respectively. Each 25 µL reaction contained 12.5 µL absolute QPCR SYBR green 2 x reaction mix (Abgene, Epsom, UK), 1.25 µL of each primer (30 µM), 2.5 µL bovine serum albumin (BSA; 10 mg ml^{-1}) and 50 ng template DNA. All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). PCR conditions for *Verrucomicrobia* and total bacteria were as described by Fierer et al. (2005) with the modification of annealing temperature (60^oC) and forward primer (Ver53) in the case of *Verrucomicrobia*. PCR amplifications and product quantification were performed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Automated analysis of PCR amplicon quality (for example, PCR baseline subtraction, Ct-threshold setting to the linear amplification phase) and quantity was performed with ROTOR-GENE 6 software (Corbett Research, Sydney, Australia). Statistical analyses of qPCR data were performed using the STATISTICA 10 package (StatSoft Inc, Tulsa, OK, USA).

One-way ANOVA was used to determine the significance of the differences between forest and deforested soil samples. The comparison of soil samples was based on *post hoc* analysis using Tukey's HSD test.

3.2.6 Verrucomicrobial 16S rRNA gene profiling by T-RFLP

T-RFLP analysis was used to characterize the verrucomicrobial community structure in soils from forest and deforested sites considering each of 30 DNA samples. Verrucomicrobial 16S rRNA gene fragments were amplified in duplicate 25 mL reactions using the primers VMB537f (5'-GCCAGCAGCCGCGGTAATACA-3') (O'FARREL; JANSSEN, 1990) and 1378r (HEUER et al., 1997). The forward primer was labeled with hexacarboxifluorescein at the 5'end. Each 25 mL reaction mixture contained 2.5 mL reaction buffer 10 X (Invitrogen, Carslad, CA, USA), 0.75 mL $MgCl_2$ (50 mM), 0.5 mL of each primer (10 μM), 0.2 U of Platinum Taq DNA Polymerase (Invitrogen), 0.5 μL of each dNTP (1 mM), 10 ng of bovine serum albumin (BSA; 10 mg ml^{-1}) and 10 ng template DNA. The following conditions were chosen for amplification after optimization: initial denaturation for 5 min at 95°C; 35 cycles of 1 min at 95°C, 30 s at 60°C, and 1.5 min at 72°C; and a final extension for 10 min at 72°C. The duplicate hexacarboxifluorescein-labeled PCR products for each sample were pooled and purified using GFX^{TM} PCR DNA and the Gel Band Purification Kit (Amersham Pharmacia Biotech, NJ, USA) after agarose gel electrophoresis. Purified products were aliquotted into three separate tubes (175 ng each tube) for 15 mL digestion reactions with 10 U of the restriction enzymes *Alu*I, *Msp*I and *Hha*I (Invitrogen, Carlsbad, CA) for 3 h at 37^{o}C. Fluorescently labeled terminal restriction fragments (TRF) were separated and detected using an ABI PRISM 3100 Genetic Analyzer capillary sequencer (Applied Biosystems). Before injection, the samples were precipitated using sodium acetate/EDTA, and denatured in the presence of 10 mL formamide and 0.25 mL GS-500 ROX size standard (Applied Biosystems). TRF profiles were evaluated using the Peak Scanner (Applied Biosystems) and T-REX (http://trex.biohpc.org/) software packages. TRFs were defined by aligning peaks using a clustering threshold (range specified equal to 1.0). TRFs shorter than 50 bp or contributing less than 0.5 % of the total TRF signal were excluded. Matrices (concatenated matrix with the three enzymes) for presence/absence were analyzed using CANOCO 4.5 (ter BRAAK; ŠMILAUER, 2002) to generate ordinations of T-RFLP profiles by principal coordinate analysis (PCA). A distance matrix (Jaccard metric) was

constructed from presence/absence data, and this similarity matrix was used for ANOSIM statistics to test the hypothesis that soil verrucomicrobial community structure was altered by deforestation. The magnitude of the ANOSIM's *R* values indicates the degree of separation between two communities, with a score of 1 indicating complete separation and 0 indicating no separation. Calculation of similarity coefficient and ANOSIM were carried out using Primer six (version 6.1.5, Primer-E Ltd., Plymouth, UK).

3.2.7 High-throughput sequencing of soil metagenome

High-throughput sequencing was performed by Macrogen Inc. Company (South Korea) using Illumina HiSeq 2000 based on aliquots from the same DNA samples used for 16S rRNA gene pyrosequencing. The sequencing strategy was index PE101+8+101 cycle (Paired End sequencing, 101-bp reads and 8-bp index sequence). Files containing 2.3 to 13.9 Gb of sequences were generated for the soil DNA samples. The shotgun sequencing is available through the Metagenomics Rapid Annotation (MG-RAST) server found online (http://metagenomics.anl.gov) under project accession 'Amazon soil metagenome 1' and accession numbers 4493649.3 (FS_1A), 4493544.3 (FS_1C), 4493545.3 (FS_1E), 4493892.3 (FS_2A), 4493546.3 (FS_2C), 4493893.3 (FS_2E), 4493547.3 (FS_3A), 4493548.3 (FS_3C), 4493549.3 (FS_3E), 4493402.3 (DS_1A), 4493493.3 (DS_1C), 4493498.3 (DS_1E), 4493650.3 (DS_2A), 4493651.3 (DS_2C), 4493652.3 (DS_2E), 4493653.3 (DS_3A), 4493654.3 (DS_3C) and 4493655.3 (DS_3E).

3.2.8 Functional annotation of soil metagenomic sequences and statistical analysis

Low-quality bases (quality score lower than 20) were trimmed from both ends using the Phred algorithm with seqtk (https://github.com/lh3/seqtk). Paired end data was concatenated into a single file and unassembled DNA sequences were annotated using MG-RAST pipeline version 3.2. Sequences were identified using BlastX with 50 bp as minimum alignment length and $E<1x10^{-5}$ as *E*-value cut-off (DINSDALE et al., 2008; SMITH et al., 2012). A subsystem-based approach to genome annotations as implemented in the SEED platform was utilized to help reveal metabolic pathways (OVERBEEK et al., 2005). Firstly, sequences were filtered for the *Bacteria* domain and metabolic profiles were generated using the bacterial sequences matches to the SEED database. Secondly, the relative abundance of

each functional category (SEED subsystem classification) was estimated in each shotgun-sequenced sample by comparing the number of sequences classified as belonging to a specific functional category versus the number of classified functional sequences per sample. One-way ANOVA was used to determine significance of the difference between forest and deforested soils regarding to each functional category in shotgun sequencing samples from all the three sampling areas.

3.2.9 Network analysis

For bacteria-bacteria-soil (BBS) associations network and function-function-soil interactions (FFS) associations network Spearman's correlation coefficients were calculated between relative abundances of bacterial groups or functional categories and soil factors for forest soils or deforested soils by using the "multtest" package in R (version 2.6.0, The R Foundation for Statistical Computing). Cut-off of coefficient $r > 0.75$ and significant positive and negative correlations (P values < 0.01) were considered. The topology of both BBS and FFS network graphs was made based on average path length (parameter Network Interpretation: directed) (BRANDES, 2001), and modularity (parameter randomize) (BLONDEL, 2008) in order to determine the number of nodes in each network. Gephi open-source software (BASTIAN et al., 2009) was applied for analysis and visualization of the BBS and FFS networks.

3.3 Results

3.3.1 Soil physicochemical characteristics

Based on ANOSIM of the soil chemical properties, forest and deforested soils formed significantly distinct groups for all sampling sites, as indicated by significant R values (Area 1, $R = 0.680$ and $P<0.001$; Area 2, $R = 0.760$ and $P<0.001$; Area 3, $R = 0.488$ and $P<0.001$). Organic matter, base saturation, moisture content and factors linked to soil acidity such as Al and Al saturation were significantly ($P<0.05$) higher in forest soils as compared to adjacent deforested soils for all three sampling areas (Table 1). Soil pH, however, was not significantly ($P>0.05$) different between forest and deforested soils.

Table 1 - Soil chemical factors of the 0- to 20-cm topsoil layer at forest and deforested sites from three discontinuous areas (real replication) in the Southeastern Brazilian Amazon

Soil factors	Area 1		Area 2		Area 3	
	Forest site	Deforested site	Forest site	Deforested site	Forest site	Deforested site
pH	4.76a[(1)] ± 0.3	5.02a ± 0.1	4.9a ± 0.4	5.32a ± 0.1	4.6a ± 0.2	5.26a ± 0.1
OM	54.8a ± 5.1	39.6b ± 3.6	58.6a ± 21.6	39.8b ± 4.1	31.8a ± 1.8	15.8b ± 8.7
P	1.0a ± 0	1.4a ± 0.9	1.0a ± 0	1.2a ± 0.4	4.8a ± 0.8	5.8a ± 1.6
K	0.9a ± 0.1	0.46b ± 0.2	0.7a ± 0.1	0.44a ± 0.3	0.4a ± 0.05	0.34a ± 0.05
Ca	2.0a ± 0.7	3.8a ± 1.6	1.4a ± 0.5	12.2b ± 6.2	2.4a ± 2.6	9.6b ± 6.3
Mg	2.0a ± 0.7	2.0a ± 1.0	1.4a ± 0.5	4.6b ± 2.2	1.0a ± 0	8.8b ± 6.8
Al	20.6a ± 4.0	12.0b ± 1.9	21.8a ± 11.7	7.6b ± 4.0	12.4a ± 1.1	3.6b ± 1.8
H+Al	89.4a ± 8.6	70.0b ± 5.6	102.4a ± 40.9	57.6b ± 8.5	61.4a ± 4.7	40.4b ± 6.9
M	81.0a ± 5.8	65.4b ± 11.8	83.6a ± 9.3	33.2b ± 17.6	78.2a ± 10.1	21.2b ± 15.6
SB	4.8a ± 1.4	6.32a ± 2.4	3.48a ± 0.9	16.9b ± 8.4	3.74a ± 2.6	18.8b ± 13.2
V	5.0a ± 1.4	8.0b ± 2.4	3.8a ± 1.9	22.4b ± 8.8	5.8a ± 3.5	30.0b ± 15.7
CEC	94.0a ± 9.6	76.2b ± 7.4	106.2a ± 40.6	74.6a ± 9.9	65.1a ± 5.6	59.2a ± 10.4
B	0.3a ± 0.04	0.2a ± 0.1	0.3a ± 0.07	0.2a ± 0.07	0.2a ± 0.01	0.2a ± 0.01
Cu	0.3a ± 0.05	0.2b ± 0.04	0.3a ± 0.04	0.3a ± 0.08	0.2a ± 0.07	0.2a ± 0.04
Fe	228.6a ± 62.4	85.6b ± 17.0	227.4a ± 96.5	77.8b ± 10.0	120.0a ± 32.3	76.2a ± 38.4
Mn	4.0a ± 3.0	2.0a ± 0.6	1.7a ± 0.9	3.3a ± 1.4	1.6a ± 0.4	0.6b ± 0.1
Zn	0.3a ± 0.1	0.3a ± 0.05	0.2a ± 0.07	0.2a ± 0.05	0.3a ± 0.08	0.3a ± 0.2
H	28.66a ± 4.3	24.38b ± 1.5	30.0a ± 6.3	23.09b ± 1.6	21.90a ± 1.0	18.50b ± 0.7

FS, soil from forest site; DS, soil from deforested site. 1, 2 and 3 indicate different sampling areas (Supplementary Table 1).
Ca, Mg, K, Al, potential acidity (H+Al), sum of base (SB) are expressed in $mmol_c.kg^{-1}$; OM is expressed in $g.kg^{-1}$; P is expressed in $mg.kg^{-1}$; B, Fe, Mn, Zn, K, Cu and cation exchange capacity in pH 7 (CEC) are expressed in $mg.dm^{-3}$. P-K – Mehlich 1 extractor. Ca-Mg-Al – KCl 1N. H+Al – SMP extractor. OM – organic matter. m – Al saturation index. SB – sum of bases. V – base saturation index. H – moisture content.
(1) The values are averages based on quintuplicate sampling points in each site. Standard deviations are shown in the table.
(2) Values with the same letters were not significantly different ($P<0.05$) based on upon a Tukey's HSD test. Tukey's test was performed separately for each of the three sampling sites regarding to soil samples taken from forest and deforested areas.

3.3.2 Deforestation effect on the relative abundance of bacterial groups

The abundance of most bacterial groups, as judged by sequence recovery and RDP 2-based classification (http://rdp.cme.msu.edu/), was not statistically different between forest and deforested soils (Table 2). The two most dominant groups, *Acidobacteria* and *Alphaproteobacteria* did not differ in relative abundance among sampled areas. However, the relative abundance of *Verrucomicrobia* (total community) and *Verrucomicrobia* (*Spartobacteria*) was significantly different between forest and deforested soils in all replicates of the three sampling areas (Area 1, $P<0.005$; Area 2, $P<0.005$; Area 3, $P<0.0005$) (Table 2). The relative abundance of *Actinobacteria* was statistically significant between forest and deforested soils in Area 2 ($P<0.005$) and Area 3 ($P<0.005$). Other bacterial groups showed statistically different abundances ($P<0.005$) across forest versus deforested soils in only Area 3. Groups of bacteria such as *Chlamydiae*, *Planctomycetes*, *Verrucomicrobia* (total community) and *Verrucomicrobia* (*Spartobacteria*) showed higher relative abundance in forest soils than deforested soils (Table 2). The relative abundance of *Actinobacteria* was higher in deforested soils than forest soils in the three sampling areas. The phylum *Verrucomicrobia* represented on average 5% and 2.5% of the bacterial sequences from forest and deforested soils, respectively, of which *Verrucomicrobia* (*Spartobacteria*) was the most abundant, accounted for 83% and 72% of the total verrucomicrobial community in forest and deforested soils, respectively.

Redundancy analysis (RDA) of the relative abundance of the bacterial taxonomic groups showed that *Verrucomicrobia* (*Spartobacteria*) and non-classified *Verrucomicrobia* were associated with forest soils, as were several other groups of bacteria (Figure 1). However, *Verrucomicrobia* (*Opitutae*), *Acidobacteria* subgroups 4, 6, 7, 10, 13, 16 and 18, *Actinobacteria*, and many others were more closely related to deforested soils than forest soils characteristics (Figure 1).

There were significant differences ($P<0.05$) in 16S rRNA gene copies between soils from forest and adjacent deforested sites regarding to the relative and absolute proportions of the verrucomicrobial community in each of three replicates of sampling area (Table 2). In general, the relative proportion of *Verrucomicrobia* declined in response to deforestation from approximately 5% to 2.5%.

Table 2 - Abundance of soil bacterial groups based on pyrosequencing analysis and quantitative real-time PCR measurements in three discontinuous areas in the Southeastern Brazilian Amazon regarding to forest (FS) and deforested (DS) sites

	Area 1		Area 2		Area 3		Statistics
	Forest site	Deforested site	Forest site	Deforested site	Forest site	Deforested site	FS *vs.* DS
Relative abundance (Pyrosequencing analysis)							
Acidobacteria	22.0[(1)]a	19.7a	16.0a	14.7a	21.6a	16.1b	ns[(2)]
Acidobacteria Gp1	11.5	10.9	7.8	6.3	11.2	6.5	ns
Acidobacteria Gp2	5.4	4.3	3.9	3.5	4.8	4.0	ns
Acidobacteria Gp3	4.7	4.3	3.8	4.4	5.3	5.0	ns
Actinobacteria	9.6a	11.4a	10.4a	16.4b	9.7a	18.7b	**
Bacteroidetes	1.6a	1.2a	1.6a	2.2a	0.3a	1.6b	ns
Chlamydiae	0.2a	0.2a	0.2a	0.1a	0.4a	0.1b	*
Firmicutes	1.5a	1.1a	1.9a	2.5a	0.5a	2.1b	ns
Gemmatimonadetes	0.4a	0.3a	0.5a	0.7a	0.2a	0.9b	ns
Planctomycetes	2.5a	1.7a	2.1a	1.6a	3.5a	2.6b	*
Alphaproteobacteria	26.2a	30.9a	30.9a	28.1a	30.2a	21.1b	ns
Betaproteobacteria	4.7a	4.5a	6.5a	6.2a	1.8a	3.5b	ns
Deltaproteobacteria	2.0a	2.0a	2.7a	1.9a	2.2a	1.6b	ns
Gammaproteobacteria	2.6a	2.9a	3.4a	3.2a	2.5a	1.3b	ns
Proteobacteria;Other	5.1a	6.4a	4.9a	4.6a	6.9a	2.9b	ns
Verrucomicrobia (total)	5.3a	3.3b	3.6a	3.0b	5.2a	1.9b	***
Verrucomicrobia (*Spartobacteria*)	4.5a	2.5b	2.7a	1.0b	4.7a	1.7b	***
Verrucomicrobia (*Opitutae*)	0.1a	0.1a	0.1a	0.2a	0.0a	0.0a	ns
Verrucomicrobia (subgroup 3)	0.6a	0.6a	0.7a	0.8a	0.4a	0.2b	ns
Verrucomicrobia (other)	0.1a	0.1a	0.1a	0.1a	0.1a	0.0b	ns
Other[(3)]	16.3a	14.5a	15.2a	14.6a	15.2a	25.6b	ns
Absolute abundance (qPCR assays) (10^7 16S rDNA copies g soil)							
Total bacteria	14.2[(4)]a (±0.7)	11.9a (±3.7)	13.3a (±2.1)	11.3a (±4.4)	18.0a (±2.0)	15.7a (±0.6)	ns[(2)]
Verrucomicrobia	0.56a (±0.01)	0.32b (±0.06)	0.61a (±0.07)	0.29b (±0.01)	0.76a (±0.01)	0.30b (±0.01)	**
Relative abundance (qPCR assays) (%)							
Verrucomicrobia	3.9a (±0.42)	2.7b (±0.62)	4.6a (±0.33)	2.6b (±0.22)	4.2a (±0.5)	1.9b (±0.66)	**

[(1)]Average for each of three replicates soil. Phylum with abundance <1% is not shown. [(2)]Tukey's HSD test was performed considering FS *vs.* DS regarding to all sampling sites. Significance levels: ns: $P>0.05$; ** $P<0.005$, *** $P<0.0005$. [(3)]Other non-classified bacteria according to RDP database. [(4)]Average for each of five replicates soil.

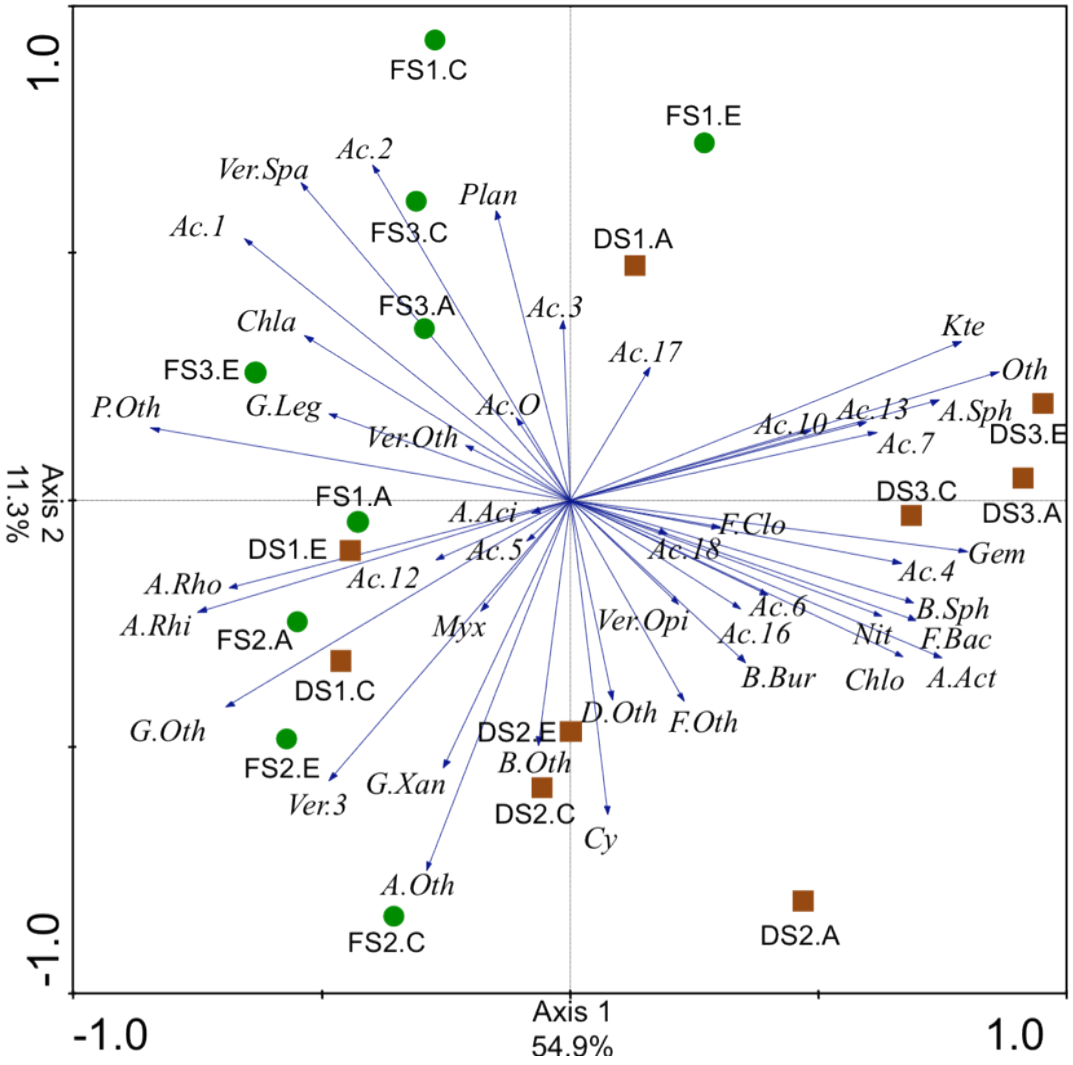

Figure 1 - Constrained ordination diagram for sample plots (● = forest soils; ■ = deforested soils) in the first two redundancy analysis (RDA) axes based on the soil chemical characteristics of the different sampling areas and their relationship with the relative abundance of bacterial groups. Each vector points to the direction of increase for a given bacterial group and its length indicates the strength of the correlation between this variable and the ordination scores. Symbols refer to individual replicates (A, B, C, D and E) of the following sampling sites: forest site located at Area 1 (FS1), Area 2 (FS2) and Area 3 (FS3); deforested site located at Area 1 (DS1), Area 2 (DS2) and Area 3 (DS3). Labels according to taxonomic affiliation (see legend text - Figure 5). Soil factors were labeled according to described in item 2.2

3.3.3 Effect of deforestation on verrucomicrobial community structure

The 16S rRNA marker gene yielded complex verrucomicrobial community fingerprints, consisting of dominant peaks and numerous peaks of lesser intensity. PCA based

on T-RFLP data from restriction profiles generated by the three enzymes revealed differences between the structure of verrucomicrobial community in deforested versus intact forest soils. PCA ordination based on T-RFLP data explained 48% (first two axes) of the included variance, and showed distinct groups for forest and deforested sites (Figure 2). This grouping pattern was confirmed by a significant *R*-value ($R = 0.819$, $P = 0.001$) verified by ANOSIM based on the presence and absence of terminal restriction fragments in T-RFLP profiles. Soil samples from deforested sites were distinctly grouped in the ordination according to the sampling sites ($R = 0.610$, $P = 0.001$). However, verrucomicrobial community structure did not exhibit any grouping by sampling site in forest soils ($R = 0.173$, $P = 0.001$).

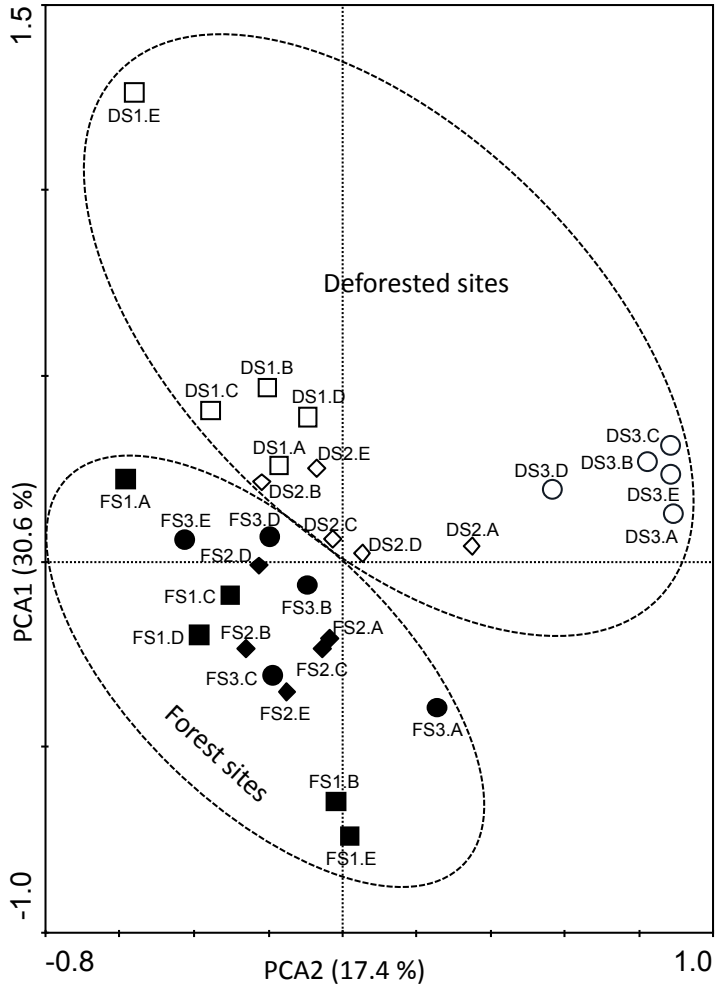

Figure 2 - Principal coordinate analysis-plot based on the structure of soil verrucomicrobial communities as determined by terminal restriction fragment length polymorphism (T-RFLP) analysis. Symbols refer to individual replicates (A, B, C, D and E) of the following sampling sites: black squares, forest site located at Area 1 (FS1); black diamonds, forest site located at Area 2 (FS2); black circle, forest site located at Area 3 (FS3); no-filled squares, deforested site located at Area 1 (DS1); no-filled diamonds, deforested site located at Area 2 (DS2); no-filled circle, deforested site located at Area 3 (DS3)

3.3.4 Overview of functional approach based on the metagenomic data

Shotgun sequencing of soil DNA from three real replications of mature forest and adjacent deforested sites in the Amazon rainforest resulted in approximately 473 million sequences (227 million from forest soils and 246 million from deforested soils) after quality-based filtering procedure. Of those, approximately 322 million sequences were distributed in taxonomic domains (*Archaea*, *Bacteria*, *Eucaryota* and *Viruses*), with 266 million sequences classified as belonging to *Bacteria* domain (129 million from forest soils and 137 million from deforested soils). Subsystem-based approach revealed that protein metabolism, carbohydrates and amino acids and derivatives bacterial categories represented higher relative numbers of sequencing reads in forest than in deforested soils (Figure 3). However, only DNA and protein metabolism categories of bacterial functions were significantly different accounted for forest versus deforested soils (Figure 3), as less bacterial groups perform DNA metabolism in deforested soils (Figures 4A and 4B). Genes indicating functions that were found not to be different quantitatively between forest and deforested soils turned out to belong to different bacterial guilds for the different soils. In these guilds, *Firmicutes* played a dominant role in deforested soils and they were absent in forest soils. *Verrucomicrobia* were the dominant bacteria together with *Actinobacteria* and *Proteobacteria* in forest soils (Figures 4C - 4H).

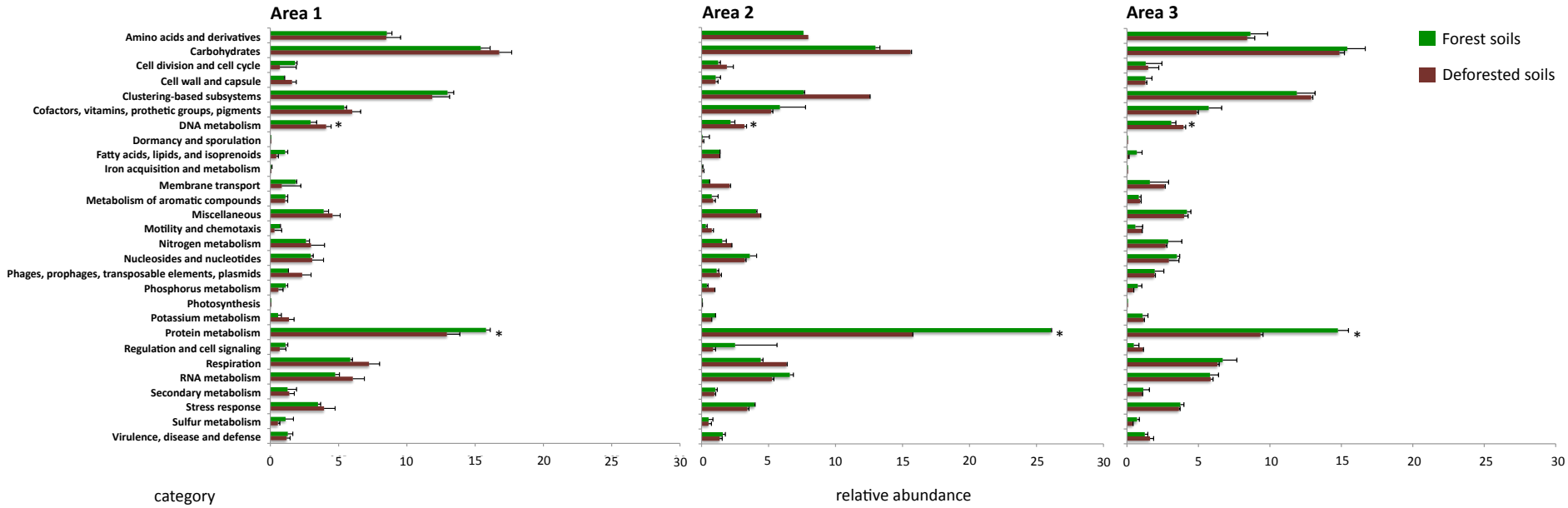

Figure 3 - Relative abundance of functional categories (SEED subsystem category) in bacterial community based on metagenome data. Percentage of total sequencing reads in each SEED subsystem categories is presented here. The error bars show calculated standard variation of triplicate samples and * indicate a statistical significant with a *P*-value <0.05 in a Tukey's HSD test. Statistical significant was indicated only when difference between forest and deforested soils regarding to a functional category was revealed for all of three sampling areas

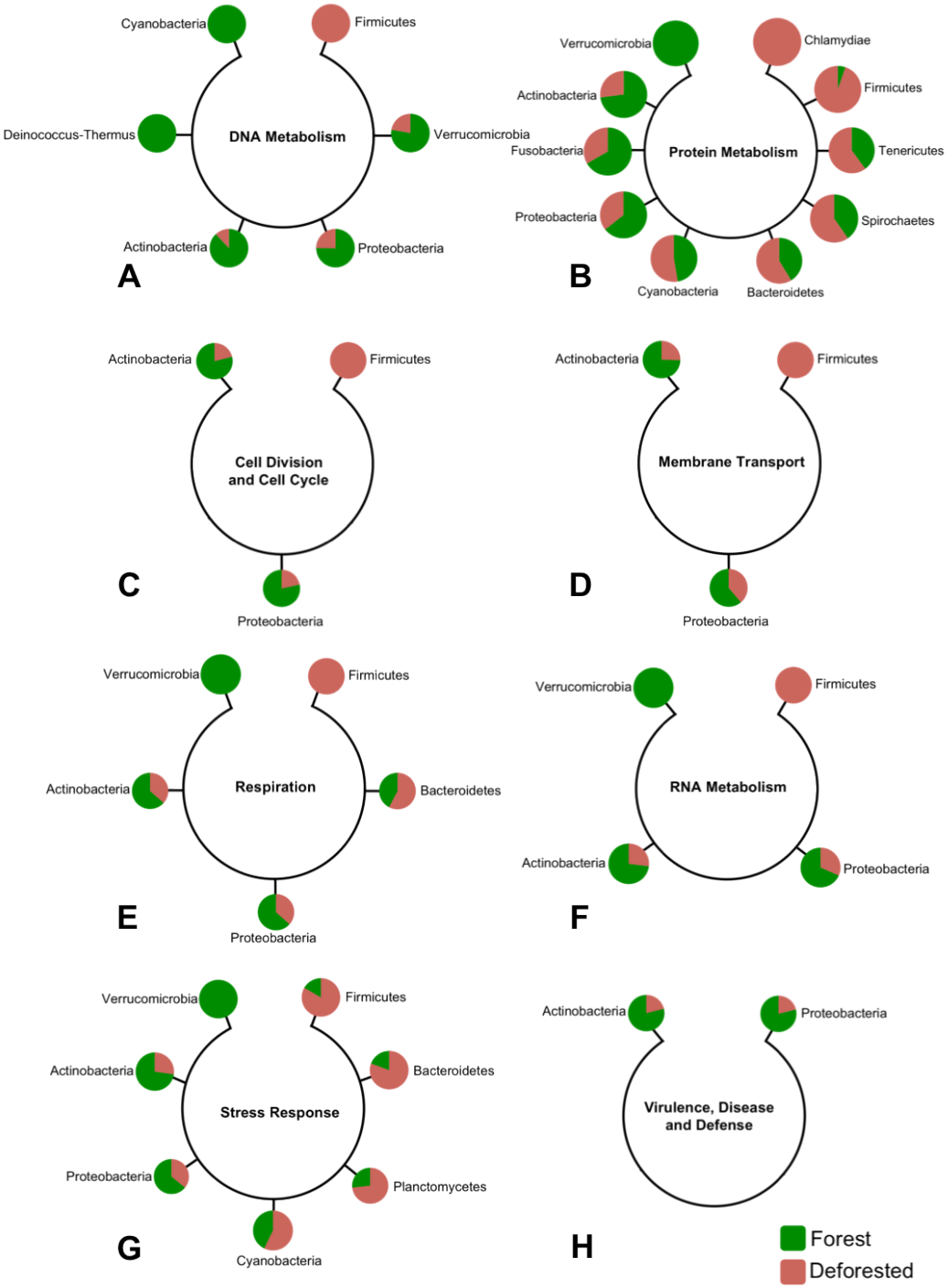

Figure 4 - Bacterial guilds for (**A**) DNA metabolism, (**B**) protein metabolism, (**C**) cell division and cell cycle, (**D**) membrane transport, (**E**) respiration, (**F**) RNA metabolism, (**G**) stress response and (**H**) virulence, disease and defense estimated in forest and deforested soils by accounting the number of bacterial sequences classified as belonging to each of eight functional categories

3.3.5 Network analysis

The BBS association network in forest soils (Figure 5A) was markedly different that constructed for adjacent deforested soils (Figure 5B). In general, the forest soil BBS network was more complex than the association network from deforested soils. In forest soils, the BBS network had 50 nodes and 117 edges, and the modularity was 0.456 with 7 communities. In adjacent deforested soils, the BBS network had 43 nodes and 93 edges, with a modularity of 0.597 and 4 communities.

The BBS networks of forest soils and deforested soils showed different patterns of association among bacterial groups and between bacterial groups and abiotic soil factors (Figures 5A and 5B). The BBS network of deforested soils (Figure 5B) comprised higher number of bacteria-bacteria associations (*i.e.*, without association with abiotic soil factors) than BBS network of forest soils (Table 3). However, the number of bacteria-soil associations (*i.e.*, association between bacterial groups and abiotic soil factors) was higher in BBS network of forest soils than in BBS network of deforested soils.

In opposition to the results revealed by BBS association networks, deforested soils revealed more complex FFS association network than forest soils (Figures 6A and 6B). In FFS networks, more function-function associations and function-soil factors associations were revealed for deforested soils than for forest soils (Figure 6A and 6B; Table 3). In forest soils, the FFS network had 30 nodes and 27 edges, and the modularity was 0.746 with 9 communities. In adjacent deforested soils, the FFS network had 51 nodes and 82 edges, with a modularity of 0.593 and 5 communities.

Table 3 - Number of correlations as inferred by Spearman based on 16S rRNA-based pyrosequencing, functional metagenomic approach based on shotgun DNA sequencing and soil factors measurements

Correlations	Forest soils			Deforested soils		
	Positive	Negative	Total	Positive	Negative	Total
bacteria-bacteria-soil	62 (53%)	55 (47%)	117	54 (58%)	39 (42%)	93
bacteria-bacteria	33 (53%)	34 (62%)	67 (57%)	44 (80%)	25 (64%)	69 (74%)
bacteria-soil	29 (47%)	21 (38%)	50 (43%)	11 (20%)	14 (36%)	25 (26%)
	Positive	Negative	Total	Positive	Negative	Total
function-function-soil	08 (30%)	19 (70%)	27	48 (58%)	34 (42%)	82
function-function	08 (100%)	11 (58%)	19 (70%)	31 (65%)	27 (79%)	58 (71%)
function-soil	0 (0%)	08 (42%)	08 (30%)	17 (35%)	07 (21%)	24 (29%)

The total numbers of pairwise correlations ($r>0.75$) as well as significant correlations ($P<0.01$) are shown for forest soils and deforested soils.

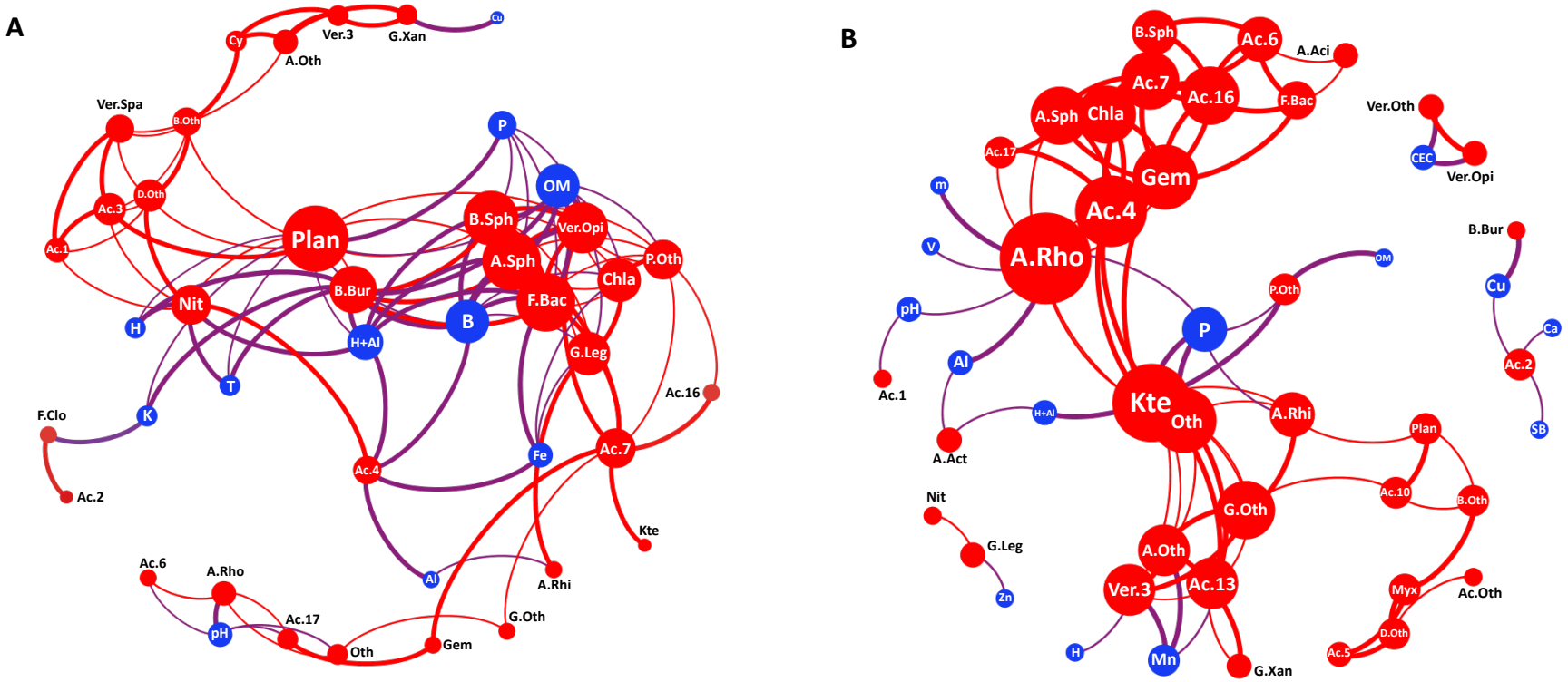

Figure 5 - Bacteria-bacteria-soil (BBS) association network of (**A**) forest and (**B**) adjacent deforested soils in Brazilian Amazon. The size of each node is proportional to the number of connection (that is, degree). The tick lines represent positive correlations and thin lines represent negative correlations. Soil factors were labeled according to described in Navarrete et al. (2012). Bacterial groups were labeled according to taxonomic affiliation as follow: Ac.1, *Acidobacteria* subgroup 1; Ac.2, *Acidobacteria* subgroup 2; Ac.3, *Acidobacteria* subgroup 3; Ac.4, *Acidobacteria* subgroup 4; Ac.5, *Acidobacteria* subgroup 5; Ac.6, *Acidobacteria* subgroup 6; Ac.7, *Acidobacteria* subgroup 7; Ac.10, *Acidobacteria* subgroup 10; Ac.13, *Acidobacteria* subgroup 13; Ac.16, *Acidobacteria* subgroup 16; Ac.17, *Acidobacteria* subgroup 17; Ac.Oth, non-classified *Acidobacteria*; A.Aci, *Actinobacteria* (*Acidimicrobiales*); A.Act, *Actinobacteria* (*Actinomycetales*); A.Oth, *Alphaproteobacteria* (non-classified *Alphaproteobacteria*); A.Rhi, *Alphaproteobacteria* (*Rhizobiales*); A.Rho, *Alphaproteobacteria* (*Rhodospirillales*); A.Sph, *Alphaproteobacteria* (*Sphingomonadales*); B.Bur, *Betaproteobacteria* (*Burkholderiales*); B.Oth, *Betaproteobacteria* (non-classified *Betaproteobacteria*); B.Sph, *Bacteroidetes* (*Sphingobacteria*); Chla, *Chlamydiales*; D.Oth, *Deltaproteobacteria* (non-classified *Deltaproteobacteria*); F.Bac, *Firmicutes* (*Bacilli*); F.Clo, *Firmicutes* (*Clostridia*); Gem, *Gemmatimonadetes*; G.Leg, *Gammaprotebacteria* (*Legionellales*); G.Oth, *Gammaproteobacteria* (non-classified *Gammaproteobacteria*); G.Xan, *Gammaproteobacteria* (*Xanthomonadales*); Kte, *Ktedonobacteria*; Myx, *Deltaproteobacteria* (*Myxococcales*); Nit, *Nitrospira*; Plan, *Planctomycetes* (*Planctomycetacia*); P.Oth, *Proteobacteria* (non-classified *Proteobacteria*); Ver.Opi, *Verrucomicrobia* (*Opitutae*); Ver.Spa, *Verrucomicrobia* (*Spartobacteria*); Ver.3, *Verrucomicrobia* subgroup 3; Ver.Oth, *Verrucomicrobia* (non-classified *Verrucomicrobia*); Oth, other non-classified bacteria according to RDP database.

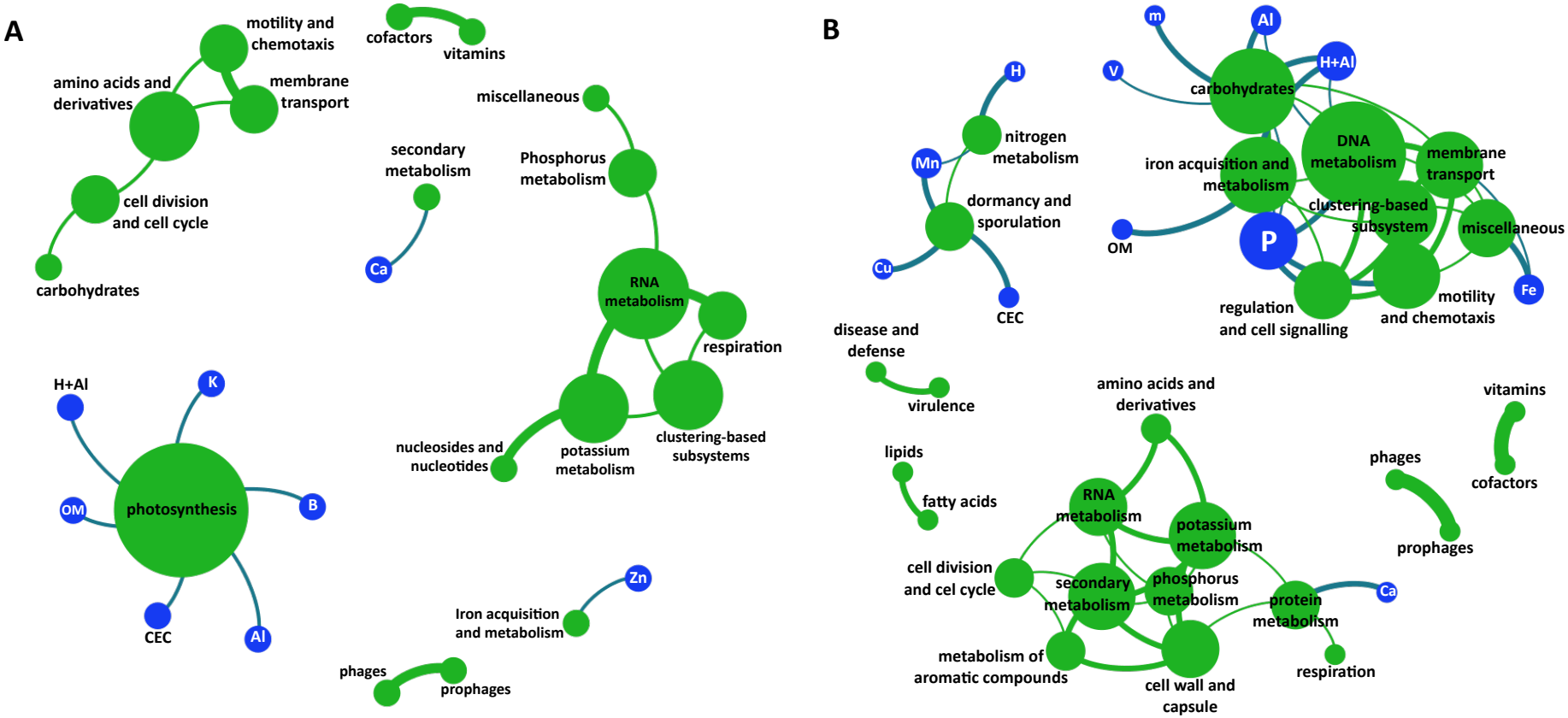

Figure 6 - Function-function-soil (FFS) association network of (**A**) forest and (**B**) adjacent deforested soils in Brazilian Amazon. The size of each node is proportional to the number of connection (that is, degree). The tick lines represent positive correlations and thin lines represent negative correlations. Soil factors were labeled according to described in Navarrete et al. (2012). Functional subsystems were labeled according to SEED subsystems category

3.4 Discussion

The dynamics of deforestation in the Brazilian Amazon are complex (BARONA et al., 2010), but there are certain distinct patterns. Local farmers practice slash-and-burn of the forest in the Southeastern Brazilian Amazon, *i.e.*, clearing of a primary forest followed by burning, in order to support cropping systems such as annual and semi-annual crops (agriculture). This practice generally occurs in the presence of an abundant and dry fuel load (*e.g.* plant residues such as trunks and branches of trees). Numerous findings on the effects of fire on soil properties are available in the literature. Burning has generally been shown to result in extensive nutrient losses following deforestation (LIKENS et al., 1970; BOYLE, 1975; MROZ et al., 1985; PATRICK; SMITH, 1975). As reviewed by Certini (2005), wildfires cause significant removal of organic matter, and marked alteration in both the density and composition of microbial and soil-dwelling invertebrate communities. In a tropical deciduous forest soil, effects of burning on microbial degradation of organic matter were assigned to both soil heating and chemical changes (GARCIA-OLIVA et al., 1999).

The lack of organic substrate input due to the absence of vegetation cover may have contributed to lower organic matter levels in deforested soils. Moreover, the loss of a proper microenvironment in the soil due to deforestation can lead to the collapse of the decomposition process and this, ultimately resulting in reduced organic carbon and other nutrient levels in the soil (BASU; BEHERA, 1993). Rapid losses of soil nutrients through leaching in the absence of vegetation cover have also been reported by Mroz et al. (1985) and Prasad et al. (1994). Declines in the moisture level of the deforested soils were also indicative of alterations in the soil hydrological regimes of deforested sites. The reduced soil moisture content in deforested soils might be due to the evaporation caused by the direct exposure of soil surface to incoming radiation (LAL, 1989). These changing in soil chemical properties due deforestation were also verified in our sampling areas located at Amazon region.

As a response to these drastic changes we found more complex association networks involving bacterial taxonomic groups and abiotic soil factors in forest soils as compared to deforested soils. However, the network linking functional categories to abiotic soil factors was more complex in deforested soils as compared to forest soils. We conceptualize that bacterial life in deforested soils has been and still is under more stress, resulting in reduced complexity of taxonomy-based networks. The opposite behavior of the function-based network may point to an adaptation process directed towards the maintenance of sufficient

functional diversity under conditions of environmental disturbance. Interestingly, more positive linkage associations among bacterial taxonomic groups were showed in deforested soil network as compared to forest soil network. This may also reflect adaptation processes at bacterial community levels under deforestation, where the lower concentrations of nutrients and organic matter urge for more cooperation in bacterial guilds.

To date, no studies had previously focused on responses of bacterial taxonomic groups to soil factors associated to deforestation in Amazon forest. In this study, different groups of bacteria were assessed from soil and our results revealed the phylum *Verrucomicrobia* as a sensitive indicator of deforestation effects in Amazonian soils. The distribution of the *Verrucomicrobia* phylum in soils (including plant rhizosphere soils) is variable and apparently extremely sensitive to changes in the environment (KIELAK et al., 2008; THOMPSON et al., 2011). *Verrucomicrobia* has a widespread distribution, and is known to be one of the most common and diverse phyla in soil habitats (ZHANG; XU, 2008; BERGMANN et al., 2011), including those of the Amazon (BORNEMAN; TRIPLETT, 1997; KIM et al., 2006; NAVARRETE et al., 2010). Via the use of cultivation-independent approaches, clear differences in the abundance of the *Verrucomicrobia* phylum were observed between two different regions in the Brazilian Atlantic forest (BRUCE et al., 2010; FAORO et al., 2010); among four regions of the Americas (ROESCH et al., 2007); and between forest and grassland soils in Germany (NACKE et al., 2011). Buckley and Schmidt (2001) and Lipson and Schmidt (2004) also showed that the *Verrucomicrobia* were sensitive to seasonal changes. The functional significance of density declines and community shifts of *Verrucomicrobia* are yet unknown, but our results point out this phylum as an important member of bacterial guilds in forest soils regarding to bacterial functions as stress response, respiration and RNA metabolism.

Although the functional significance of some changes in microbial community is yet unclear in soil habitat, in this study the shotgun assembly approach revealed that DNA and protein metabolism of soil bacteria were differently accounted in forest and deforested soils. Genetic material is highly dynamic, and genome stability is constantly challenged by endogenous and environmental agents that induce DNA lesions, genome rearrangements and other types of genotoxic stress. The balancing act between these forces has a major impact on the fitness of cells. In soils from deforested sites considered in this studied, probably many

bacterial genomes are beholden to the rapid generation of genome instability as a means of adaptation to changes in soil characteristics due forest clear-cutting and burning.

Thus, we conclude that deforestation of Amazon forest soils simplifies association networks among different bacterial taxonomic groups and abiotic soil factors. Furthermore, deforestation led to a consistent decline in the abundance of *Verrucomicrobia* and alterations in verrucomicrobial community structure. In order to adapt to this condition function-based associations network were enhanced, indicating a higher degree of risk spreading for the maintenance of soil functioning.

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4 ACIDOBACTERIAL COMMUNITY RESPONSES TO AGRICULTURE MANAGEMENT OF AMAZON FOREST SOILS[1]

ABSTRACT

This study focused on the impact of land use changes in the Amazon area on the size and composition of the acidobacterial community in order to obtain sufficient insight in these dynamics for the development of microbial bio-indicators as early-warning systems for agricultural soil management effects in this tropical region. Taxon-specific quantitative real-time PCR (qPCR) assays and pyrosequencing-based analysis of the V4 16S rRNA gene region were applied to study the acidobacterial community in bulk soil samples from croplands and adjacent native forests as well as in the rhizosphere of soybean. Based on qPCR measurements, *Acidobacteria* accounted in average of 23%, 18% and 14% of the total bacterial signal, in forest soils, cropland soils and soybean rhizosphere samples, respectively. A total of 175,434 16S rDNA sequences were affiliated to the *Bacteria* domain in twelve independent soil samples. The phylum *Acidobacteria* represented on average 28%, 16% and 17% of the sequences generated from the forest soils, cropland soils and soybean rhizosphere samples, respectively, of which *Acidobacteria* subgroup 1 was dominant among the 26 acidobacterial subgroups classified. Subgroups 2-8, 10, 11, 13, 17, 18, 22 and 25 were also detected. Operational taxonomic units (OTUs) were assembled using 97% similarity for each *Acidobacteria* subgroup and revealed significantly higher numbers in subgroups 4, 6 and 7 in cropland soils than in forest soils. Subgroups 4, 6 and 7 respond to decrease of Aluminium in soil, however subgroups 6 and 7 respond to high content of Ca, Mg, Zn, P, Fe, Mn and B in soil. The results showed differential response of the *Acidobacteria* subgroups to abiotic soil factors, and indicated acidobacterial subgroups as potential early-warning bio-indicators of agricultural soil management effects in the Amazon area.

Keywords: soil microbiology; soil factors; land-use changes; tropical rainforest; 16S rRNA gene

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[1] NAVARRETE, A.A.; KURAMAE, E.E.; de HOLLANDER, M.; PIJL, A.S.; van VEEN, J.A.; TSAI, S.M. Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. **FEMS Microbiology Ecology**, Amsterdam, 2012. DOI: 10.1111/1574-6941.12018. Appendix B – Full text.

4.1 Introduction

Soil bacterial communities in the Amazon area have been analyzed in different types of soils (BORNEMAN; TRIPLETT, 1997; KIM et al., 2007; O'NEILL et al., 2009; CENCIANI et al., 2009; JESUS et al., 2009; NAVARRETE et al., 2010). Based on these studies, the bacterial community composition was revealed in soils from different Amazon regions. The *Acidobacteria* phylum has been described as dominant in soils from Western Amazon (KIM et al., 2007; JESUS et al., 2009) and Central Amazon (NAVARRETE et al., 2010). However, the role of this dominant group in the bacterial community of Amazon soils is largely unknown.

Acidobacteria have consistently been detected in many different habitats around the globe by 16S rRNA gene-based molecular surveys, including soil and rhizosphere niches (CHOW et al., 2002; KUSKE et al., 2002; GREMION et al., 2003; QUAISER et al., 2003; FIERER et al., 2005; STAFFORD et al., 2005; JANSSEN 2006; SANGUIN et al., 2006; De CÁRCER et al., 2007; SINGH et al., 2007; KIELAK et al., 2009; DeANGELIS et al., 2009). These observations have revealed that Acidobacteria are ubiquitous and among the most abundant bacteria phyla in soil. In spite of their high abundance, little information is available on their ecology, which is mainly due to the lack of culturable representatives in bacterial collections (KISHIMOTO et al., 1991; LIESACK et al., 1994; COATES et al., 1999; BRYANT et al., 2007; EICHORST et al., 2007; FUKUNAGA et al., 2008; KOCH et al., 2008; LEE et al., 2008; NUNES da ROCHA et al., 2009; WARD et al., 2009; KULICHEVSKAYA et al., 2010; PANKRATOV; DEDYSH, 2010; EICHORST et al., 2011; MÄNNISTÖ et al., 2011; PANKRATOV et al., 2011).

Land-use changes is one of the greatest threats to biodiversity worldwide, and one of the most devastating land-use changes, especially in the tropics, is the conversion of intact forests into cultivation fields (MORTON et al., 2006). The Amazon is a vast, yet vulnerable, hotspot of biodiversity, and the maintenance of soil fertility is critical to sustaining these diverse ecosystems. With this in mind, increased attention has recently been paid to belowground biodiversity in the Amazonian region, and a number of cultivation-independent studies have sought to assess the impact of land-use changes on microbial communities resident to Amazon soils (BORNEMAN; TRIPLETT, 1997; CENCIANI et al., 2009; JESUS et al., 2009; NAVARRETE et al., 2010; 2011). However, despite this increased appreciation of belowground microbial diversity in the Amazonian region, little is still known about bacterial

taxa responses to alterations in soil chemical properties and fertility in consequence to deforestation and agricultural management of Amazon forest soils.

Because of the substantial effects that land-use changes may have on the chemical and physical characteristics of former tropical forest soils, and the high abundance and presumed importance of *Acidobacteria* for the functioning of soil systems, we would like to obtain better insight into the ecological characteristics of *Acidobacteria* in Amazon soils and in soils recently converted into cultivation. For this purpose, we applied quantitative real-time PCR and pyrosequencing spanning the V4 region of the 16S rRNA gene to analyze the abundance and the composition of the acidobacterial community inhabiting bulk soil from soybean croplands and adjacent forests collected in an agricultural zone located in the Southeastern Brazilian Amazon as well as soybean rhizosphere soil from mesocosm experiment. The relative abundances of *Acidobacteria* at the phylum and subgroup taxonomic levels in soil samples were correlated with soil factors in order to explore responses to alterations in soil characteristics due land-use changes and agriculture management of Amazon forest soils.

4.2 Materials and methods

4.2.1 Site description and soil sampling

Bulk soil samples were collected in two different field locations in the Southeastern Brazilian Amazon, in the state of Mato Grosso, Brazil in the Porto dos Gaúchos municipality (-15^{o}13’39” S and -54^{o}04’31” W) and the Ipiranga do Norte municipality (-13^{o}21’57” S and -54^{o}54’24” W) (Figure 1). Oxisol is the predominant soil order in the sampling sites (SEPLAN, 2001), and the climate in the region is classified as Am (Koppen’s classification), with annual average temperature of 28 ^{o}C and average precipitation of 2000 mm.

The field locations were considered replicates and the sampling sites were selected according to the vegetation cover, soil use and management practices. In the Porto dos Gaúchos municipality, areas covered with native tropical rainforest were cleared in 2008 and subsequently converted into agricultural land. Since 2004, forest conversion to agricultural use occurred in areas located in Ipiranga do Norte municipality. In both field locations, forest

conversion to agricultural use followed annually the rotational production order: millet – soybean – maize, under no-tillage. After deforestation, fertilizers, pesticides and a liming treatment were applied to the cropland fields of both locations. The cropland fields received different amounts of lime to increase soil pH to 5 and 6.

Bulk soil samples were collected from soybean production fields before sowing the seeds (October 2009) and after soybean (*Glycine max* [L.] Merrill cultivar M-SOY 8866) harvest (April 2010) in order to consider an expected variation in soil characteristics during the soybean cultivation (Figure 1). Soil samples were also collected at the same time from adjacent forests to represent the native soil–plant conditions (Figure 1). At each sampling site, the soil samples were collected from five points. One central sampling point and other four sampling points (at least 50 m apart from the central point) directed towards the north, south, east and west of the central point. Soil samples were taken from the 0 to 20 cm topsoil layer (tilled zone). First, the litter layer was removed, and then the soil sample was collected using a 5 cm (diameter) aseptic cylindrical core. A total of 40 bulk soil samples were collected in field (2 field locations x 2 sampling sites per field location x 2 sampling periods x 5 sampling points per site). Samples were transported to the laboratory under ice, stored at −20 °C until processing within 72 h after sampling.

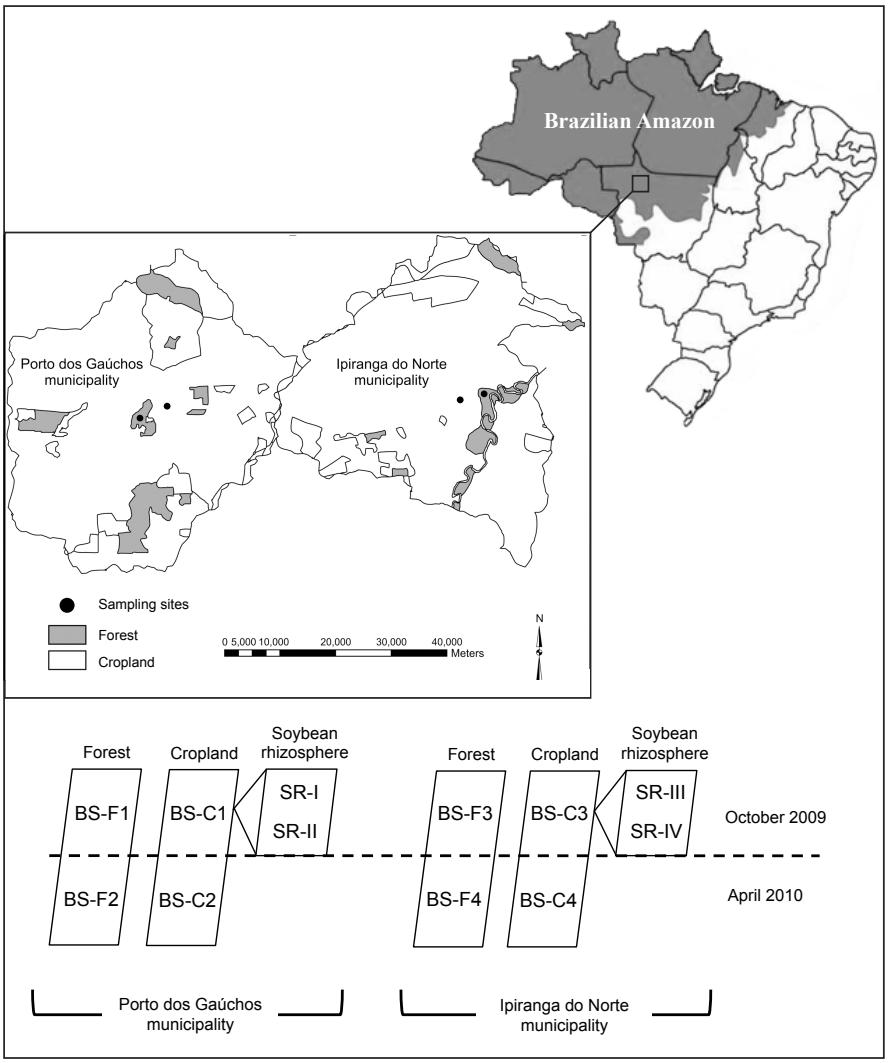

Figure 1 - Location of the sampling sites in the Southeastern Brazilian Amazon and time-location scheme for soil sampling (bulk soil from field and soybean rhizosphere from greenhouse mesocosm experiment). Sampling points were located under cropland and adjacent forest in both the Porto dos Gaúchos municipality (-15°13'39" S and -54°04'31" W) and the Ipiranga do Norte municipality (-13°21'57" S and -54°54'24" W), state of Mato Grosso, Brazil. Bulk soil sampling was performed before sowing soybean seeds (October 2009) and after soybean harvest (April 2010) in cropland sites. Concomitantly, bulk soil samples were taken in adjacent forests. In the time-location scheme for soil sampling codes indicate the name attributed to the pooled DNA samples used for pyrosequencing analysis (BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere). The roman numerals I, II, III and IV indicate replication of greenhouse mesocosm experiment

4.2.2 Abiotic soil factors

For soil physicochemical analysis, soil sample consisted of a composite sample for each sampling site and period by mixing five sub-samples collected from the sampling points. From each composite sample, a sub-sample was removed, air dried and passed through a 2 mm mesh sieve, and then analyzed according to EMBRAPA (1997) at the Department of Soil Science at the University of São Paulo (ESALQ-USP) for the soil fertility properties. Soil pH was measured in a 1:2.5 soil:water suspension. Exchangeable Al, Ca and Mg were extracted with KCl 1 mol L^{-1}. Calcium and Mg were determined by atomic absorption spectrometry and Al by acid-base titration. Phosphorous and K were extracted by ion exchange resin. Potential acidity (H + Al) was estimated by an equation based on the pH determined in SMP buffer solution (pH SMP). Available micronutrients (Fe, Mn, Zn, and Cu) were extracted by Mehlich 1 and determined by atomic absorption spectrometry. Boron was extracted with hot water and determined by spectrophotometry with azomethine-H at 420 nm. Some of the results allowed the calculation of other parameters-such as: exchangeable bases (SB), the sum of Ca, Mg and K; cation exchange capacity (CEC), the sum of Ca, Mg, K, Al, and H; base saturation (V), the percentual relation of SB and CEC; and Al saturation (m%), the percentual relation of exchangeable Al and CEC. Soil texture was determined with Bouyoucos densimeter after shaken the soil vigorously with NaOH 1 mol L^{-1} as dispersant.

4.2.3 Statistical analysis for soil factors

Analyses of similarity (ANOSIM) of soil physicochemical properties were performed considering the differences in cropland and forest soils. Distance matrix (Euclidean metric) was constructed with non-transformed data. ANOSIM was carried out using Primer six (version 6.1.5, Primer-E Ltd., Plymouth, UK). Correlation analysis was performed in R (version 2.6.0, The R Foundation for Statistical Computing). Spearman's rank coefficients were calculated to investigate the correlation among the soil factors.

4.2.4 Soybean rhizosphere soil

In order to consider the abundance and composition of acidobacterial community in the soybean rhizosphere niche, normalizing the influence of environmental parameters (such as moisture regime and temperature) on the growth conditions for the plants, soybean plants were grown in mesocosms in the greenhouse at CENA-USP, Piracicaba, São Paulo. Soil samples were taken at the same five sampling points used for bulk soil collecting in soybean production fields before sowing the seeds. Each soil sample consisted of a composite sample by mixing five sub-samples collected from the 0 to 20 cm topsoil layer. Twenty mesocosms in ceramic pots (30 cm high x 20 cm diameter) were filled with the soils. Duplicate pots were filled with 8 kg of soil and disposed over a bottom of 5-cm layer filled with washed stones. Seeds of the same soybean cultivar (M-SOY 8866) used in field were germinated in the mesocosms at a regime of 12/12 h light/dark cycle and average temperatures 28 ^{o}C at day and 19 ^{o}C at night. The moisture content of the pots was regularly adjusted by an external visual controller installed in each pot. The temperature and moisture regimes were chosen close to natural conditions to create optimal growth conditions for the plants. The plants were grown from November 2009 until January 2010 (84 days). A total of 20 mesocosm samples from soybean rhizosphere soil were taken in late January, prior to plant maturity (soil from 2 soybean production fields x 5 sampling points per field x 2 ceramic pots per sampling point). Roots and associated soil were transported to the laboratory on ice and then processed to obtain the rhizosphere soil. The roots were shaken to remove the loose soil, and the tightly attached soil including small aggregates (<0.5 cm) was used for DNA extraction.

4.2.5 Isolation of DNA from soil and quantitative real-time PCR assay

DNA was extracted from 250 mg of bulk soil samples from croplands and adjacent native forests, and mesocosm samples from soybean rhizosphere soil using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. DNA extraction was performed in triplicate for each soil sample and stored at −20 °C until use. Quantitative real-time PCR (qPCR) using the 16S rRNA gene as biomarker was performed to assess the abundance of the acidobacterial and total bacterial communities in each of 40 bulk soil samples and 20 mesocosm samples from soybean rhizosphere soil. As standard, amplicons of *Acidobacteria capsulatum* (DSMZ 11244) and bacterial clone from an environmental sample were obtained by PCR using primers

pA-F (5'-AGAGTTTGATCCTGGCTCAG-3') (EDWARDS et al., 1989) and 1378R (5'-CGGTGTGTACAAGGCCCGGGAAGG-3') (HEUER et al., 1997), purified (QIA-quick PCR purification kit, Qiagen, Venlo, the Netherlands) and ligated into the pGEM-T vector (Promega, Leiden, the Netherlands). Ligation products were transformed with *E.coli* JM109 competent cells (Promega, Leiden, The Netherlands). Cloned inserts were re-amplified using primers SP6 and T7, and plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Venlo, the Netherlands) from appropriate clones (i.e. belonging to the desired target group). DNA standard curves were generated by dilution series of 10^3 to 10^8 copies μL^{-1} using duplicate 10-fold dilutions of isolated plasmid DNA. For qPCR of 16S rRNA gene fragments from *Acidobacteria* and total bacteria: Acid31 (5'-GATCCTGGCTCAGAATC-3') (BARNS et al., 1999)/Eub518 (5'-ATTACCGCGGCTGCTGG-3') (MUYZER et al., 1993) and Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') (LANE, 1991)/Eub518 primer pairs were used, respectively. Each 25 µL reaction contained 12.5 µL absolute qPCR SYBR green 2 x reaction mix (Abgene, Epsom, UK), 1.25 µL of each primer (30 µM), 2.5 µL bovine serum albumin (BSA; 10 mg ml^{-1}) and 50 ng template DNA. All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). PCR conditions for *Acidobacteria* and total bacteria were carried out as described by Fierer et al. (2005) with the modification of annealing temperature to 49 ^oC for *Acidobacteria*. PCR amplifications and product quantification were performed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Automated analyses of PCR amplicon quality (for example, PCR baseline subtraction, Ct-threshold setting to the linear amplification phase) and quantity were performed with ROTOR-GENE 6 software (Corbett Research, Sydney, Australia). Statistical analyses of qPCR data were performed using the STATISTICA 9 package (StatSoft Inc, Tulsa, OK, USA). One-way ANOVA was used to determine significance of the differences between all soil samples. The comparison of soil samples was based on *post hoc* analysis using Tukey's HSD test. Correlations were calculated to test the relationships between acidobacterial relative abundance and soil factors by using the "multtest" package in R (version 2.6.0, The R Foundation for Statistical Computing).

4.2.6 Amplification and sequencing of 16S rRNA gene fragments

Primers targeting the V4 region of bacterial 16S rRNA gene were used for the amplification. Adapter sequence was added to the primers as recommended by Roche; barcodes of 8 bp and AC linker (added to forward primers only); and the primer sequence 5'-AYTGGGYDTAAAGNG-3' for the forward primer and 5'-CCGTCAATTCMTTTRAGT-3' for the reverse primer (http://pyro.cme.msu.edu/). DNA template in PCR reactions consisted of pools by mixing aliquots from 15 DNA sub-samples from bulk soil (three replicates of soil DNA extraction for each of five soil samples) for each sampling site and period or 15 DNA sub-samples (three replicates of soil DNA extraction for each of five soybean rhizosphere soil samples) for each replicate of mesocosm experiment. PCR reaction was performed in duplicate for each pooled DNA sample (samples were described in Figure 1). The reactions contained 2.5 × reaction buffer, 0.2 mM of each dNTP, 1 μM of each primer (Alpha DNA, Montreal, Canada), 10 ng of template DNA, and 0.056 U of FastStart High-Fidelity PCR System enzyme blend (Roche Applied Sciences, Indianapolis, IN, USA). The following conditions were chosen for amplification after optimization: initial denaturation for 5 min at 95 °C; 27 cycles of 45 s at 95 °C, 45 s at 57 °C, and 1.5 min at 72 °C; and final extension for 10 min at 72 °C. After gel analysis, amplicons from 10 reactions were combined and purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). PicoGreen base quantification allowed the normalization for construction of multiplex amplicon pools (HARRIS et al., 2010). The samples were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA).

4.2.7 Sequence analysis

Sequences and quality information were extracted from the Standard Flowgram Format (SFF) files using the SFF converter tool in the Galaxy interface (GOECKS et al., 2010). Sequences were analyzed with the Qiime version 1.2.1 scripts (CAPORASO et al., 2010), which were made available in the Galaxy interface. Firstly, all the sequences that had a perfect match to the primer sequences contained no homopolymer run exceeding six nucleotides and without ambiguous characters were assigned to samples by matching to barcode sequences. Reverse complementary sequences were put in the forward orientation

and assigned to samples by partially matching to barcode sequences. This step was confirmed by V-REVCOMP tool using Hidden Markov Models (HMMs) (HARTMANN et al., 2011). Secondly, sequence errors introduced during pyrosequencing were detected using the option for titanium data in the Denoiser 0.91 program (REEDER; KNIGHT, 2010). The sequences were also checked for PCR chimeras using UCHIME version 4.2.40 (EDGAR et al., 2011). Only sequences with a length between 200 and 350 base-pairs were accepted and bases with a quality score lower than 20 were trimmed. The low quality sequences were discarded. The obtained high quality sequences were clustered into Operational Taxonomic Units (OTUs) using UCLUST version 1.2.21 (EDGAR, 2010) with a minimum sequence identity cutoff of 97 %. For each OTU the most abundant sequence was selected as a representative for all sequences within an OTU. Taxonomy was assigned to the representative sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4), with a minimum support threshold of 60 %. Finally, the OTU table was filtered for specific taxonomic terms using scripts provided by Qiime. Relative abundances of *Acidobacteria* were estimated in each bacterial community by comparing the number of sequences classified as belonging to the *Acidobacteria* versus the number of classified bacterial sequences per sample. The relative abundance of *Acidobacteria* subgroups across all individual samples, in turn, was estimated by comparing the number of sequences classified as belonging to each *Acidobacteria* subgroup versus the number of classified acidobacterial sequences. The classified acidobacterial sequences were deposited in MG-RAST under accession numbers 4497240.3 to 4497251.3 (list of public access codes can be found in Supplementary Table 1). Explicit relationship between relative abundance of *Acidobacteria* subgroups and bulk soils factors from cropland and forest sites was examined by constrained ordination generated by redundancy analysis (RDA) performed using CANOCO 4.5 (ter BRAAK; ŠMILAUER, 2002). Spearman's rank correlation coefficients were calculated to explore the relationship between relative abundance of *Acidobacteria* subgroups and soil factors referent to different sampling sites by using the "multtest" package in R (version 2.6.0, The R Foundation for Statistical Computing). *P* values were corrected for multiple testing, using the false discovery rate controlling procedure (BENJAMINI; HOCHBERG, 1995).

4.3 Results

4.3.1 Bulk soil physicochemical factors

The soils contained 43–50 % sand, 4–6 % silt and 46–53 % clay in all sites. Based on ANOSIM of the physicochemical factors, bulk soil from cropland and adjacent forests formed distinct groups, which groups were confirmed by significant *R*-value (R=0.955, $P<0.0001$). Soil pH ranged from 3.7 to 4.7 in forest soils and from 5.0 to 5.7 in the majority of the cropland soils. The detected levels of Al, Al saturation and Fe were respectively 10, 8 and 3 times higher in forest soils than in cropland soils, except for the bulk soil from cropland BS-C1 (Figure 1; Supplementary Table 2). Sum of bases (SB) and base saturation (V) were higher in cropland soils than forest soils (Supplementary Table 2). Results showed that soil pH was negatively correlated ($P<0.05$) with Al saturation, potential acidity, Al, Fe and Mn (Table 1). Both SB and V were negatively correlated with Al ($P<0.05$), potential acidity ($P<0.05$) and Al saturation ($P<0.0005$) (Table 1).

Table 1 - Spearman's rank correlation matrix for soil chemical attributes of the 0 to 20 cm topsoil layer at different sampling sites

Soil attributes	pH	OM	Al	H+Al	m	P	K	Ca	Mg	SB	CEC	V	B	Fe	Mn	Zn	Cu
pH	-																
OM		-															
Al	-0.850*		-														
H+Al	-0.778*	0.785*	0.857*	-													
m	-0.634*		0.785*	0.785*	-												
P						-											
K			-			0.754*	-										
Ca			-0.785*	-0.785*	-1***	0.850*		-									
Mg			-0.778*	-0.742*	-0.958***	0.801*		0.958***	-								
SB			-0.785*	-0.785*	-1***	0.850*		1***	0.958***	-							
CEC											-						
V			-0.761*	-0.833*	-0.976***	0.826*		0.976***	0.934***	0.976***		-					
B	-0.876*		0.932**	0.748*									-				
Fe	-0.814*		0.904**										0.969**	-			
Mn	-0.757*		0.855*										0.938**	0.988***	-		
Zn																-	
Cu																	-

Significant levels for the Spearman's rank coefficients are indicated at the *P<0.05; **P<0.005; ***P<0.0005 level.

4.3.2 Acidobacterial community abundance

ANOVA analyses were carried out on qPCR data targeting 16S rRNA gene fragment abundances for total bacteria as well as for *Acidobacteria*. These analyses showed significant differences between bulk soils from cropland and adjacent forest ($P<0.005$) both for the relative and absolute acidobacterial abundance (Table 2). *Acidobacteria* accounted, on average, for 23 % in forest soils, 18 % cropland soils and 14 % in soybean rhizosphere of the total bacterial signals. Although the highest *Acidobacteria* 16S rRNA gene content was observed in the bulk soils, the overall abundance of *Acidobacteria* gene copies was not statistically different between bulk soils from cropland and mesocosm samples from soybean rhizosphere (Table 2). The abundance of *Acidobacteria* relative to total bacteria 16S rRNA gene copy numbers was significantly correlated with soil organic matter (R=0.627, P=0.00001), Al (R=0.413, P=0.0004) and pH (R=-0.407, P=0.008) (Figure 2). *Acidobacteria* represented the largest portion of the bacterial community in soils with high organic matter content, Al content and acidity. Soil Ca levels (R=-0.482, P=0.001) and base saturation (R=-0.494, P=0.001) were negatively and significantly correlated with the relative abundance of *Acidobacteria*.

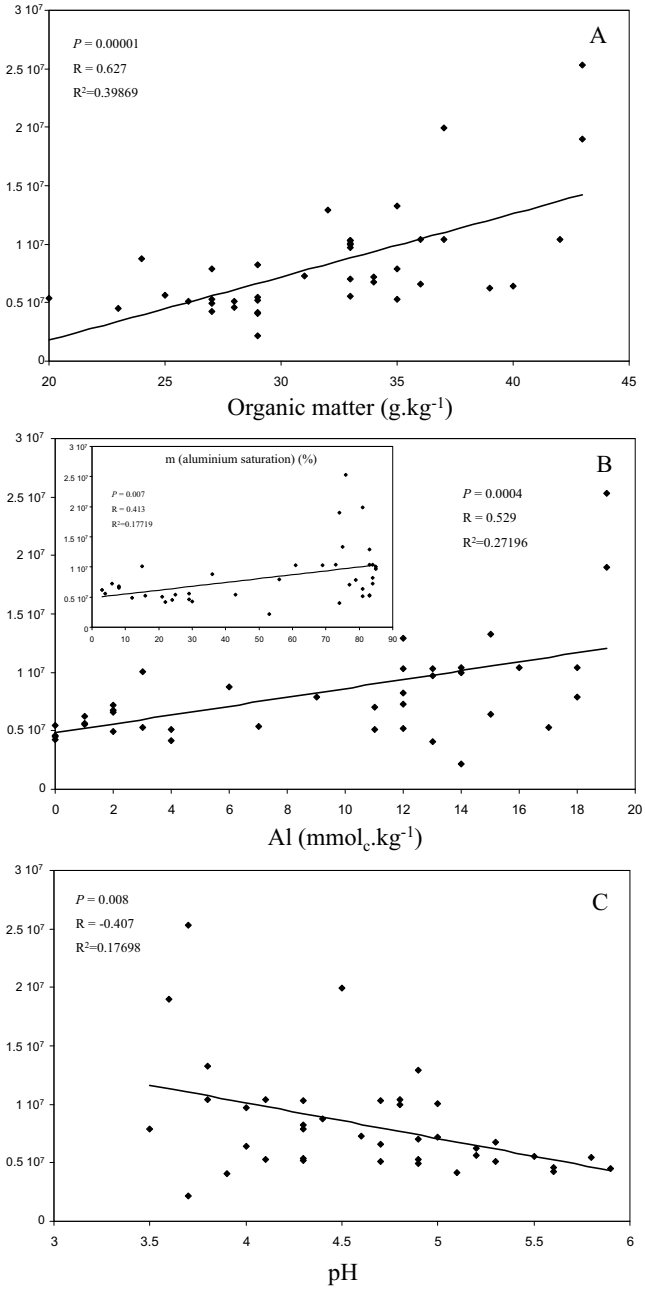

Figure 2 - Effect of (A) organic matter, (B) Aluminium (Al) and (C) soil pH on abundance of *Acidobacteria* relative to total bacteria as indicated by the number of 16S ribosomal (DNA) (rDNA) copies measured using real-time quantitative PCR assays. Spearman's rank correlations and their statistical significance are depicted. Coefficient of determination (R^2) was calculated.

Table 2 - Absolute and relative abundance of *Acidobacteria* in cropland and adjacent forest bulk soils and soybean rhizosphere niche

	Bulk soil - Forest (BS-F)				Bulk soil - Cropland (BS-C)				Soybean rhizosphere (SR)				Statistics	
	BS-F1	BS-F2	BS-F3	BS-F4	BS-C1	BS-C2	BS-C3	BS-C4	SR-I	SR-II	SR-III	SR-IV	BS-F vs. BS-C	BS-C vs. SR
Absolute abundance (qPCR analysis) (10^7 16S rDNA copies g soil)														
Total bacteria	3.48a[1] (0.17)[2]	2.90a (0.08)	6.0A (0.23)	4.59A (0.14)	1.64a (0.01)	2.96a (0.13)	7.12A (0.29)	4.55A (0.15)	2.28a (0.09)	2.89a (0.10)	7.27A (0.22)	6.08A (0.12)	ns[3] (1-2) ns (3-4)	ns (1-2) ns (3-4)
Acidobacteria	0.96a (0.07)[2]	0.84a (0.02)	1.07A (0.06)	0.85A (0.01)	0.49a (0.05)	0.56a (0.02)	0.74A (0.01)	0.50A (0.005)	0.44a (0.01)	0.45a (0.007)	0.78A (0.008)	0.72A (0.03)	** (1-2) ** (3-4)	ns (1-2) ns (3-4)
Relative abundance (%)														
Acidobacteria (qPCR assays)	27.58a (2.61)[2]	28.96a (1.83)	17.83A (2.72)	18.51A (1.76)	29.87a (2.8)	18.92b (0.12)	10.39A (2.11)	10.98A (1.18)	19.30a (1.10)	15.57a (1.64)	10.72A (1.13)	11.84A (2.53)	* (1-2) **(3-4)	ns (1-2) ns (3-4)
Acidobacteria (Pyrosequencing analysis)	19.92a (4057)[4]	22.06a (3614)	28.78A (4967)	22.97A (2771)	25.0a (2704)	15.56b (2435)	9.58A (991)	15.16A (1316)	10.43a (1848)	15.03a (3391)	7.90A (2925)	8.72A (1618)	ns (1-2) ** (3-4)	* (1-2) ns (3-4)

[1]Values with the same lower or upper-case letters were not significantly different ($P<0.05$) based on upon a Tukey's HSD test.
[2]Standard deviation of the average for each of five replicates soil.
[3]Tukey's HSD test was performed separately for field location and sampling period (samples 1-2 and 3-4). Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.005$; ***$P<0.0005$.
[4]Total number of acidobacterial reads obtained by barcoded pyrosequencing. Sequences taxonomy assignments were made using Ribosomal Database Project 2 classifier (release 10.4).

4.3.3 Phylum and subgroup-levels relative abundance of *Acidobacteria*

Sequence data were examined in soils to estimate the phylum and subgroup relative abundance of *Acidobacteria*. Across the twelve soil samples (samples described in Figure 1), *Acidobacteria* represented 19 % of all classified bacterial sequences detected (32,637 sequences out of 175,434 sequences). The relative abundance of acidobacterial sequences within an individual soil bacterial community represented on average 28 % in forest soils, 16 % in cropland soil and 17 % in soybean rhizosphere.

We further classified *Acidobacteria* DNA sequences into acidobacterial subgroups using the RDP 2 classifier (http://rdp.cme.msu.edu/). The average relative abundances of *Acidobacteria* subgroups for bulk soil from forest and cropland, and soybean rhizosphere soil samples are listed in Table 3 as well as the numbers of OTUs assembled using a 97 % similarity for each *Acidobacteria* subgroup. *Acidobacteria* subgroups 1 and 3 were the most abundant in all soils analyzed. Redundancy analysis (RDA) of the relative abundance of the *Acidobacteria* subgroups (1-7, 10 and 13) showed that some subgroups (4-7, 10 and 13) were more closely related to cropland soils (Figure 3). *Acidobacteria* subgroups 1 and 2 were more closely related to forest soils characteristics (Figure 3). Statistically significant differences were found for the abundances of the acidobacterial subgroups 4 ($P<0.05$), 6 ($P<0.05$) and 7 ($P<0.005$) relative to all *Acidobacteria* in cropland soils versus the adjacent forest soils (Table 3). Interestingly, the relative abundances of these acidobacterial subgroups (4, 6 and 7) were statistically correlated with different soil factors, specially those linked to soil acidity such as Al and Al saturation, sum of bases and base saturation (Table 4; Figure 4). Significant correlations were also found between these subgroups (4, 6 and 7) and soil Ca and Mg. The dominant *Acidobacteria* subgroup 1 was positively correlated to potential acidity and Aluminium saturation while subgroup 3 was negatively correlated to soil pH (Table 4). *Acidobacteria* subgroups 10 and 13 were detected in all three environments studied. Subgroup 10 was correlated to soil factors linked to soil acidity such as pH, Al and Al saturation. Moreover, subgroup 13 was correlated to soil P, B and Zn. Most subgroups appear to be minor constituents of soil acidobacterial communities and often occurring in one or two of the sampled soils; subgroup 11 was detected only in bulk soil from cropland; subgroup 17 was detected in bulk soil from forest and cropland, but not in soybean rhizosphere soil;

subgroups 18 and 22 were detected in bulk soil from cropland and soybean rhizosphere soil, but not in forest soils; and subgroup 25 was detected only in soybean rhizosphere soil. No sequences affiliated to *Acidobacteria* subgroups 9, 12, 14-16, 19-21, 23, 24 and 26 were detected in any soil sample (Table 3).

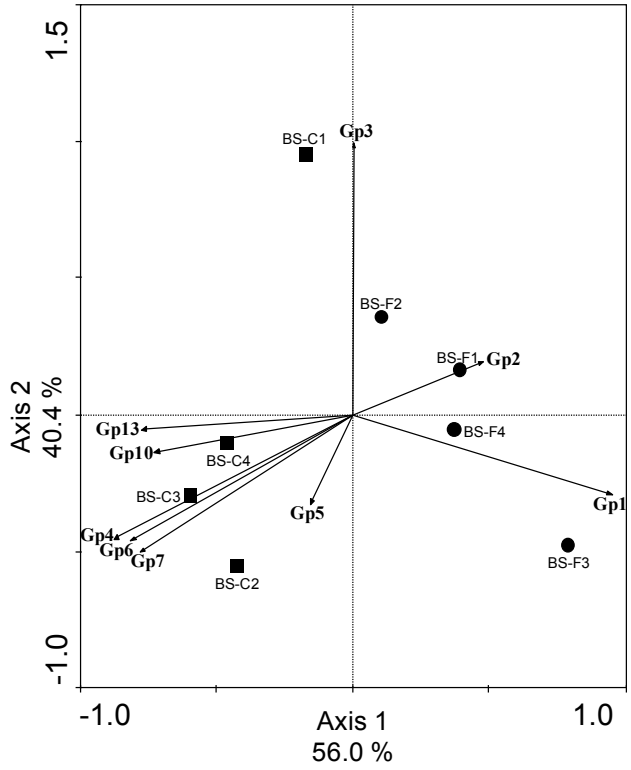

Figure 3 - Constrained ordination diagram for sample plots (● = bulk soil from forest; ■ = bulk soil from cropland) in the first two redundancy analysis (RDA) axes based on the soil chemical characteristics of the different sampling sites and their relationship with the relative abundance of *Acidobacteria* subgroups (1-7, 10 and 13). Each vector points to the direction of increase for a given acidobacterial subgroup (Gp) and its length indicates the strength of the correlation between this variable and the ordination scores. Bulk soil (BS) samples were taken before sowing soybean seeds (BS-C1 and BS-C3) and after soybean harvest (BS-C2 and BS-C4) in cropland sites. Concomitantly, bulk soil samples were taken in adjacent forests (BS-F1 and BS-F3) and (BS-F2 and BS-F4)

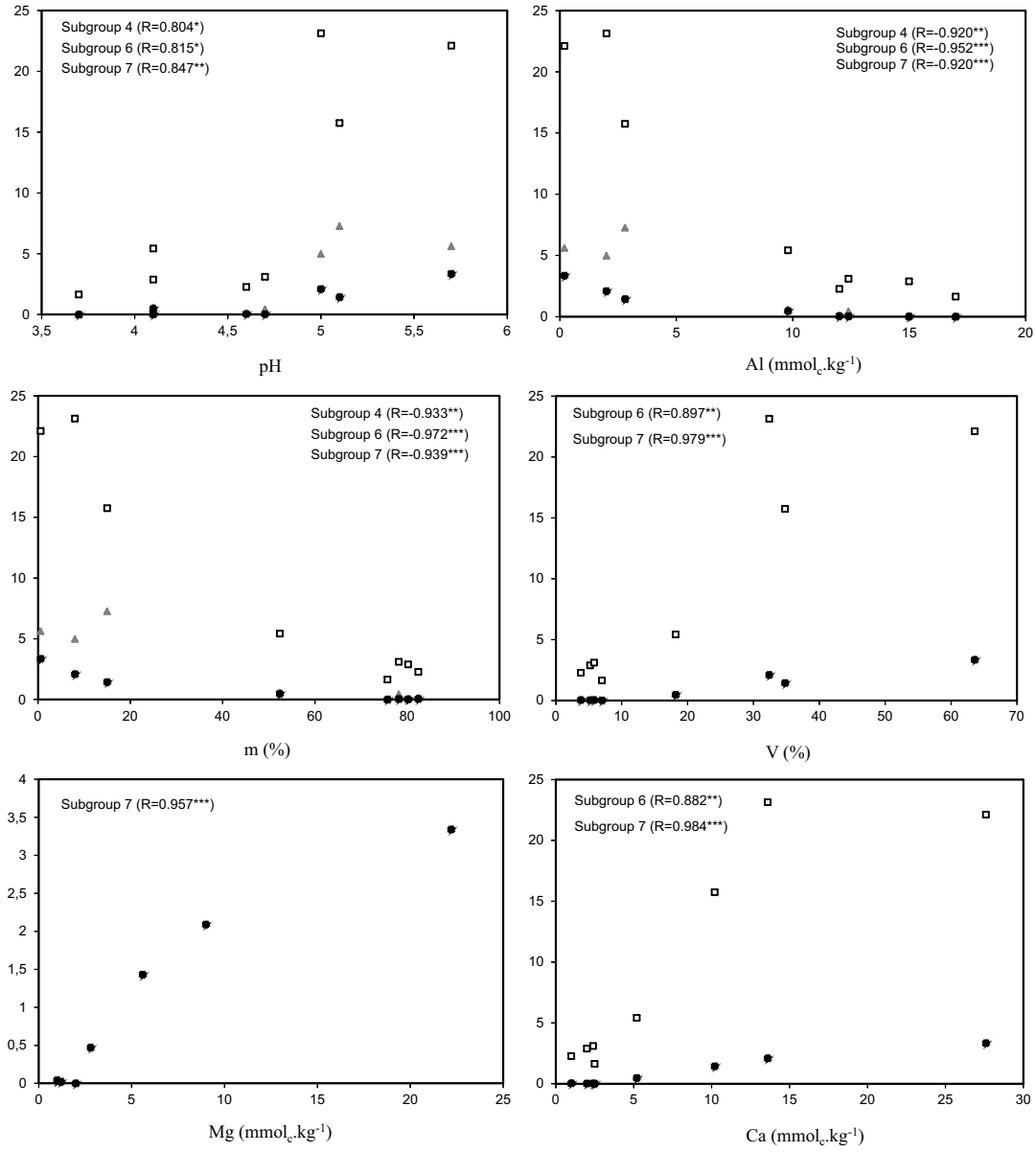

Figure 4 - Effect of soil pH, Al, Al saturation (m), base saturation (V), Ca and Mg on abundance of *Acidobacteria* subgroups (4, 6 and 7) relative to all *Acidobacteria* using pyrosequencing data. Triangles (grey) represent *Acidobacteria* subgroup 4; Squares (no filled) represent *Acidobacteria* subgroup 6; circles (black) represent *Acidobacteria* subgroup 7. Spearman's rank correlations between subgroup abundance and each soil factor are depicted. **P*<0.05; ***P*<0.005; ****P*<0.0005

Table 3 - Abundance of *Acidobacteria* subgroups relative to all *Acidobacteria* and numbers of OTUs of acidobacterial subgroups in cropland and adjacent forest bulk soils and soybean rhizosphere niche

Subgroups	% of total acidobacterial sequences (range)			OTUs (range)			Statistics (% of total acidobacterial sequences)		Statistics (OTUs)	
	BS-F	BS-C	SR	BS-F	BS-C	SR	BS-F vs. BS-C	BS-C vs. SR	BS-F vs. BS-C	BS-C vs. SR
1	51.4 (40.1-65.2)[1]	32.8 (30.9-36.5)	41.0 (29.5-60.7)	42 (41-43)	41 (34-48)	45 (41-49)	ns[2]	ns	ns	ns
2	11.2 (8.0-13.9)	7.2 (6.7-7.6)	5.3 (2.9-7.8)	16 (12-23)	16 (10-23)	12 (8-15)	ns	ns	ns	ns
3	29.0 (20.1-51.2)	29.6 (17.7-51.2)	30.8 (22.8-40.3)	27 (19-34)	32 (25-41)	39 (23-54)	ns	ns	ns	ns
4	0.1 (0.0-0.4)	4.6 (0.58-7.3)	5.4 (0.7-10.5)	2 (0-4)	18 (7-27)	11 (3-18)	* (1-2) / * (3-4)	ns	* (1-2) / * (3-4)	ns
5	4.0 (3.0-5.3)	3.8 (1.0-6.0)	1.2 (0.4-1.6)	5 (4-7)	6 (4-8)	5 (4-6)	ns	ns	ns	ns
6	2.5 (1.6-3.1)	16.6 (5.4-23.1)	13.1 (5.6-20.3)	6 (5-7)	10 (6-13)	13 (7-19)	* (1-2) / * (3-4)	ns	* (1-2) / * (3-4)	ns
7	<0.1 (0.0-0.04)	1.8 (0.5-3.3)	1.4 (0.1-2.8)	1 (0-1)	6 (4-7)	4 (1-6)	** (1-2) / * (3-4)	ns	** (1-2) / * (3-4)	ns
8	<0.1 (0.0-0.02)	<0.1 (0.0-0.07)	<0.1 (0.0-0.05)	1 (0-1)	1 (0-1)	1 (0-2)	ns		ns	
9	ND	ND	ND	ND	ND	ND				
10	0.1 (0.04-0.2)	0.3 (0.2-0.4)	0.3 (0.0-0.9)	3 (1-5)	3 (1-5)	3 (0-7)	ns	ns	ns	ns
11	ND	<0.1 (0.0-0.1)	ND	ND	1 (0-2)	ND				
12	ND	ND	ND	ND	ND	ND				
13	1.3 (0.9-1.7)	2.3 (1.5-3.3)	1.3 (0.8-1.9)	7 (5-9)	6 (5-8)	4 (3-7)	ns	ns	ns	ns
14	ND	ND	ND	ND	ND	ND				
15	ND	ND	ND	ND	ND	ND				
16	ND	ND	ND	ND	ND	ND				
17	<0.1 (0.0-0.2)	0.5 (0.0-1.3)	ND	1 (0-2)	4 (0-7)	ND	ns		ns	
18	ND	<0.1 (0.0-0.1)	<0.1 (0.0-0.2)	ND	1 (0-2)	1 (0-3)		ns		ns
19	ND	ND	ND	ND	ND	ND	ns		ns	
20	ND	ND	ND	ND	ND	ND	ns		ns	
21	ND	ND	ND	ND	ND	ND	ns		ns	
22	ND	0.1 (0.09-0.5)	<0.1 (0.0-0.02)	ND	1 (0-4)	1 (0-1)		ns		ns
23	ND	ND	ND	ND	ND	ND				
24	ND	ND	ND	ND	ND	ND				
25	ND	ND	<0.1 (0.0-0.05)	ND	ND	1 (0-1)				
26	ND	ND	ND	ND	ND	ND				

BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere.

[1]Range (%) of the average for each of four replicates soil.

[2]Tukey's HSD test was performed separately for field location and sampling period (samples 1-2 and 3-4). SR. Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.005$.

Table 4 - Spearman's rank correlation coefficients and statistical significance between abundance of *Acidobacteria* subgroups (1-7, 10 and 13) relative to all *Acidobacteria* and soil factors

Soil factors	*Acidobacteria* subgroups								
	1	2	3	4	5	6	7	10	13
pH	-0.304	-0.437	-0.707*	0.804*	0.375	0.815*	0.847**	0.710*	0.484
OM	0.347	0.558	-0.130	-0.427	0.221	-0.290	-0.375	-0.706	-0.143
Al	0.619	0.691	0.446	**-0.920****	-0.223	**-0.952*****	**-0.920*****	-0.807*	-0.669
H+Al	0.737*	0.610	0.117	-0.810*	-0.134	-0.769*	-0.826*	-0.841*	-0.465
m	0.737*	0.675	0.344	**-0.933****	-0.259	**-0.972*****	**-0.939*****	-0.723*	-0.666
P	-0.587	-0.553	-0.114	0.541	-0.069	0.705	0.478	0.352	0.802*
K	-0.410	-0.482	0.011	0.274	-0.198	0.489	0.305	-0.066	0.477
Ca	-0.530	-0.503	-0.459	0.767*	0.265	**0.882****	**0.984*****	0.543	0.330
Mg	-0.433	-0.455	-0.485	0.685	0.247	0.822*	**0.957*****	0.451	0.208
SB	0.496	-0.493	-0.469	0.736*	0.251	0.866*	**0.978*****	0.499	0.288
CEC	0.342	0.187	-0.547	-0.124	0.179	0.142	0.226	-0.540	-0.281
V	-0.608	-0.575	-0.399	0.845*	0.280	**0.897****	**0.979*****	0.653	0.402
B	0.448	0.642	0.395	-0.690	0.032	-797*	-0.666	-0.683	-0.750*
Fe	0.256	0.736*	0.327	-0.597	0.194	-0.606	-0.538	-0.686	-0.516
Mn	0.293	0.748*	0.589	-0.796*	-0.155	-0.792*	-0.673	-0.660	-0.653
Zn	-0.670	-0.326	-0.008	0.455	0.046	0.644	0.427	0.391	0.810*
Cu	-0.160	0.614	0.234	-0.242	0.356	-0.664	-0.158	-0.033	-0.038

Significant levels for the Spearman's rank coefficients are indicated at the *P<0.05; **P<0.005; ***P<0.0005 level.
Bod values indicate a significant difference using Bonferroni correction.
Coeficient of determination (R^2) for significant correlations can be found in Supplementary Table 3.

4.4 Discussion

The present study assessed the soil acidobacterial community in a Brazilian Amazon region characterized by high rate of deforestation due to the expansion of cropland (mainly soybean) into areas previously covered by native forest. Our results based on qPCR assays and pyrosequencing analyses show that the overall abundance of *Acidobacteria* (relative to all bacteria) differed between soybean cropland and adjacent native forest soils, and they revealed that the differences in soils factors were the major predictors of the variability in relative abundance of *Acidobacteria* subgroups found in these Amazon soils.

Although the overall abundance of *Acidobacteria* significantly differed ($P<0.005$) between soybean cropland and adjacent forest soils, the pyrosequencing data revealed that not all *Acidobacteria* subgroups responded to this change in land-use. Recent studies have demonstrated that changes in soil microbial communities across space are often correlated with differences in soil chemistry (FREY et al., 2004; NILSSON et al., 2007; LAUBER et al., 2008; JENKINS et al., 2009; KURAMAE et al., 2011). In particular, it has been shown that the composition, and in some cases diversity, of soil bacterial community is correlated with soil pH (SAIT et al., 2006; EICHORST et al., 2007; HARTMAN et al., 2008; JENKINS et al., 2009; LAUBER et al., 2009) and other soil factors than pH, such as Ca/Mg ratio, and Al and phosphorous content (FAORO et al., 2010). This pattern holds both for overall bacterial community composition (FIERER; JACKSON, 2006; BAKER et al., 2009; LAUBER et al., 2009; KURAMAE et al., 2010) and for the composition of individual bacterial groups (NICOL et al., 2008; DAVIS et al., 2009; JENKINS et al., 2009; JONES et al., 2009).

Although previous studies have indicated the effect of soil pH on the abundance of Acidobacteria in different soil types (JONES et al., 2009; ROUSK et al., 2010) and spatial scales (LAUBER et al., 2009), the present study shows differential responses of the *Acidobacteria* subgroups to other soil characteristics (including soil factors not measured in previous studies, such as Al, Ca, Mg, K, B and micronutrients) than soil pH altered probably by liming practice required in the agriculture management into Amazon forest soils. In the soil environment, pH is a master variable and it is related to changes in other soil factors, such as Al concentration and nutrient availability (McBRIDE, 1994). Aluminum toxicity has long been known to affect microbes as well as plants in tropical soils (WOOD, 1995; JONER et al., 2005). The soil in the region where our samples were collected are rich in Al, in particular the forest soils. Hence, it is not surprising that Al and pH would covary and correlate with

acidobacterial community in these native soils. In addition, bacterial communities dominated by *Acidobacteria* phylum changed significantly along gradients of base saturation, Al and pH in Western Amazon soils (JESUS et al., 2009).

Liming practice in agriculture is based on calculation of the necessity of Ca and Mg by the plant, tolerancy to Aluminium and clay content. In acid tropical soils, changing in soil chemical properties due lime requirement for agricultural practices includes a decrease in H^+ activity, a decrease in Al, Al saturation and Mn toxicities, an increase in Ca and Mg availability, and benefits associated with Ca as a complementary ion on the cation exchange complex (ABRUÑA et al., 1964; AMEDEE, 1976; OLIVEIRA; PAVAN, 1996). This practice appears to have direct effect on *Acidobacteria* subgroups 4, 6 and 7 since the abundance of these subgroups were negatively correlated with Al and Al saturation. However for subgroup 6, Ca and saturation of bases were additional factors, while for subgroup 7, Mg and sum of bases (K, Ca, Mg) were additional factors explaining their high abundance in cropland soils. Such elements (Mg, K, Ca) are required for the growth of all living organisms. Magnesium ions are required by large number of enzymes for their catalytic action, including all enzymes utilizing or synthesizing ATP, or those that use other nucleotides to synthesize DNA and RNA. However, the ionic magnesium can not directly be uptaken by the biological membranes because they are impermeable to magnesium (and other ions), so transport proteins must facilitate the flow of magnesium and other ions, both into and out of cells (BEVENBACH, 1990). Future studies on *Acidobacteria* subgroups 4, 6 and 7 functions certainly will elucidate the role of those subgroups in soil.

Differential responses of *Acidobacteria* subgroups to abiotic soil factors are probably due the lifestyles of these microorganisms in soils (WARD et al., 2009). When these responses are related to specific factors that are involved in soil management they may be used to develop early-warning bio-indicators for soil effects for instance due to agricultural management of Amazon forest soils into soybean cultivation. According to McCarty and Munkittrick (1996), bio-indicator is an anthropogenically-induced response in biomolecular, biochemical, or physiological parameters that has been causally linked to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization. Our findings about differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes. However, there are still not many studies focusing on responses of acidobacterial subgroups to

environmental factors. Ultimately, a better understanding of how agricultural management affects soil microbial ecology will support development of more productive, sustainable systems.

In this study, biological effects at the taxonomical acidobacterial subgroup level were detected using primer set “universal” targeting the V4 region of bacterial 16S rRNA gene. In our soil samples were detected 15 different acidobacterial subgroups among the 26 acidobacterial subgroups classified – subgroups 1-8 according to Hugenholtz et al. (1998); subgroups 9-11 according to Zimmermann et al. (2005) and subgroups 12-26 according to Barns et al. (2007). Many researchers have pointed out that not all acidobacterial subgroups could be detected using group-specific primers, such as Acid31F (BARNS et al., 1999) and ACIDO (LEE; CHO, 2011) designed for the selective amplification and detection of members of the phylum Acidobacteria. According to Barns et al. (2007), George et al. (2009), Jones et al. (2009), Kielak et al. (2009), Sait et al. (2006), Lee and Cho (2011), the two group-specific primers (Acid31F and ACIDO) do not detect the subgroups 2, 22 and 25, which subgroups were accounted in this study by using the “universal” V4 region primers. In our soil samples, acidobacterial subgroup 2 was one of the most dominant. The acidobacterial subgroups 1-8 identified in this study were also ubiquitous and abundant members of soil acidobacterial communities in soil surveys worldwide (BARNS et al., 1999; JANSSEN, 2006; JONES et al., 2009; ROUSK et al., 2010). In our analysis, acidobacterial subgroups 4, 6 and 7 showed preponderant responses to abiotic soil factors which were clearly affected by the changes in soil use from native forests into soybean production fields, such as Al, Ca, Mg, Mn and B.

In conclusion, the acidobacterial community responds to agricultural management of Amazon forest soils into soybean production sites, through the effects on various soil factors, not only those related to soil acidity. The differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes in Amazon soils recently converted into cultivation and open the possibilities to explore acidobacterial subgroups as bio-indicators for agricultural soil management effects in the wide Amazon area.

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5 MOLECULAR DETECTION ON CULTURE MEDIUM OF HITHERTO-UNCULTURED *Acidobacteria* AND OTHER BACTERIAL GROUPS FROM AMAZON SOILS

ABSTRACT

Cultivation and molecular approaches were combined in order to recover and detect *Acidobacteria* and other bacterial groups from Amazon soils on culture medium. The strategy of the cultivation procedure included the following: the use of VL55 growth medium supplemented with 0.05% of xylan as carbon source and solidified with gellan gum instead of agar; incubation in hypoxic (2% O_2 [vol/vol], 2% CO_2 [vol/vol] and 96% N_2 [vol/vol]) atmosphere for relatively long period; inclusion of aluminium potassium sulphate in growth medium; and dilution and plating of Amazon soils from primary forests, soybean cropland fields, pasture and Amazonian Dark Earth sites. From 456 colonies subjected to PCR-screening, were detected three colonies belonging to *Acidobacteria* subdivision 1 on plates inoculated with soils from soybean cropland field and pasture and one colony belonging to *Acidobacteria* subdivision 3 on plate inoculated with pasture soil (100% identity with 16S rRNA gene sequences). Culture-independent assessment based on 16S rRNA gene library comprising 437 clones confirmed that members of the phylum *Acidobacteria* only grew on plates inoculated with soils from soybean cropland field and pasture (10% and 23% of the total bacterial community, respectively). Clones in library from plates consisted majoritarily of *Proteobacteria* (orders *Burkholderiales* and *Xanthomonadales*). In conclusion, this work reports the recovery and detection on culture medium of hitherto-uncultured representatives of *Acidobacteria* subdivisions 1 and 3 from Amazon soils and the results serve as a glance at the potential to combine cultivation and molecular approaches to search for growth of *Acidobacteria* concomitantly to other bacterial groups inhabiting Amazon soils.

Keywords: soil microbiology, tropical rainforest, hypoxic atmosphere, culture-dependent approach, molecular PCR-screening

5.1 Introduction

The existence of large phylogenetic groups of bacteria that are poorly represented by cultivated strains has been revealed by culture-independent molecular surveys. Comparative phylogenetic analysis of the DNA sequences of 16S rRNA genes has shown that bacterial groups, such as the *Acidobacteria*, are ubiquitous and among the most abundant bacterial phyla in different environments, including soil (BARNS et al., 1999; HUGENHOLTZ et al., 1998; JANSSEN, 2006). In spite of their high abundance, *Acidobacteria* contains few cultured members. Of 142,153 high-quality 16S rRNA gene sequences available from bacterial isolates (Ribosomal Database Project (RDP) II v10.4; http://rdp.msu.edu/), only 141 are classified as *Acidobacteria* with cultured representatives of only 5 (subdivisions 1, 2, 3 4 and 6) of the 26 subdivisions currently exist (BARNS et al., 2007; HUGENHOLTZ et al., 1998; ZIMMERMANN et al., 2005).

The culture-independent approach based on direct recovery of bacterial 16S rRNA from tropical soils indicated the predominance of *Acidobacteria* in soils from Western Amazon (KIM et al., 2007; JESUS et al., 2009), Central Amazon (NAVARRETE et al., 2010), Southeastern Amazon (NAVARRETE et al., 2012) and Brazilian Cerrado (ARAUJO et al., 2012). However, there are not cultured representatives of *Acidobacteria* from Amazon soils. Although many laboratories are trying to obtain pure cultures of *Acidobacteria* from different environments in worldwide, the taxonomically described diversity within this group remains very limited. More than half of the currently characterized *Acidobacteria* belong to subdivision 1, and only 18/141 high-quality 16S rRNA gene sequences from bacterial isolates are classified as *Acidobacteria* subdivision 3 (Ribosomal Database Project (RDP) II v10.4; http://rdp.msu.edu/).

Members of subdivision 1 of the *Acidobacteria* have been isolated from diverse soils (JANSSEN et al., 2002; SAIT et al., 2002; JOSEPH et al., 2003; EICHORST et al., 2007; KOCH et al., 2008; STOTT et al., 2008), acidic mineral environments (KISHIMOTO et al., 1991), the termite hindgut (EICHORST et al., 2007), Sphagnum-dominated wetlands (PANKRATOV et al., 2008, 2012; PANKRATOV; DEDYSH, 2010), decaying wood (FOLMAN et al., 2008; VALÁŠKOVÁ et al., 2009) and methanotrophic enrichment culture (KOCH et al., 2008; DEDYSH et al., 2012). *Acidobacteria* subdivision 3 representatives, in

turn, have been isolated from soil (SAIT et al., 2002; JOSEPH et al., 2003; DAVIS et al., 2011; EICHORST et al., 2011) and acidic Sphagnum peat bog (DEDYSH et al., 2006; KULICHEVSKAYA et al., 2010).

In this work, we took advantage of the treatments tested in Stevenson et al. (2004) and Davis et al. (2005) combining cultivation and molecular approaches in order to recover and detect hitherto-uncultured *Acidobacteria* and other bacterial groups from Amazon soils on laboratory medium by providing high carbon concentration in culture medium, an hypoxic atmosphere (2% O_2, 2% CO_2 and 96%N_2) and longer incubation time. Given the historic conversion of forest to crop land and pasture in Amazon region (MORTON et al., 2006), and the lower organic matter content in soils under soybean cropland fields in compare to adjacent soil under mature forest in Southeastern Brazilian Amazon (NAVARRETE et al., 2012), we carried out cultivation experiment in laboratory using these soils as inoculum and other Amazon soils from pasture and Amazonian Dark Earth sites to illustrate the capacity to recover and detect in culture medium members of the division *Acidobacteria* concomitantly to other bacterial groups inhabiting different Amazon soils.

5.2 Materials and methods

5.2.1 Soil collection and manipulation

The soil samples used in this study were collected in November 2011 from six different sampling sites located at three different municipality in Brazilian Amazon region: (1) Porto dos Gaúchos municipality (-15°13’39” S and -54°04’31” W), state of Mato Grosso, Brazil, in primary forest (PF-1) and soybean cropland field (SC-1), (2) Ipiranga do Norte municipality (-13°21’57” S and -54°54’24” W), state of Mato Grosso, Brazil, in primary forest (PF-2), soybean cropland field (SC-2) and pasture (*Brachiaria brizantha*) (PA) site, and (3) Iranduba municipality (03°18'25" S and 60°32'05" W), state of Amazonas, Brazil, in an anthropogenic soil site characterized as Amazonian Dark Earth (ADE). Oxisol is the predominant soil order in the field sites located at state of Mato Grosso (SEPLAN, 2001). Soil in ADE site is classified as Hortic Anthrosol according to the World Reference Base for Soil Resources (FAO, 1998).

The composition of the bacterial community was characterized by pyrosequencing-based analysis in soils from each of six sampling sites considered in this study (NAVARRETE et al., 2012; CANNAVAN, 2011). In these soils, *Acidobacteria* and *Proteobacteria* were dominant members of the bacterial community followed by *Chloroflexi*, *Planctomycetes*, *Actinobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Verrucomicrobia*, *Nitrospira* and *Bacteroidetes*. *Acidobacteria* subdivisions 1-7, 10, 11, 13, 16-18, 22 and 25 were detected among the 26 acidobacterial subdivisions classified. *Acidobacteria* subdivisions 1, 2 and 3 were dominant in soils from forests, soybean cropland fields and pasture. In ADE site, *Acidobacteria* subdivisions 4 and 6 were dominant members. The pH value for the different soils is: forest soils (PF-1 = 3.9 and PF-2 = 4.0), cropland soils (SC-1 = 5.3 and SC-2 = 5.2), pasture soil (5.1) and Amazonian Dark Earth (5.1).

For the present study, five soil cores (5 cm diameter, 0- to 20-cm topsoil layer) were collected from each sampling site using aseptic cylindrical soil corer and transported to the laboratory in a sealed polyethylene bag at ambient temperature and stored overnight at 4 ^{o}C. At each site, the soil samples were collected from five sampling points. One central sampling point and other four sampling points (at least 50 m apart from the central point) directed towards the north, south, east and west of the central point. An accurately weighed aliquot of soil (6 g fresh weight) from each of five soil sample per sampling site was suspended in 100 mL of sterile salt solution (3.9 g 2-[*N*-morpholino]ethanesulfonic acid, 0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 0.4 mM $(NH_4)_2HPO_4$ per liter) and mixed using a Teflon-coated magnetic bar for 15 min at approximately 200 r.p.m. The resultant suspensions were serially diluted in 10-fold steps by adding 1 mL of the previous dilution to 9 mL sterile salt solution, stirring for 5 min between dilutions. Volumes of 100 μL from 10^{-5} and 10^{-6} dilutions of the soil were spread onto plates of medium VL55 (see below) using a sterile glass spreader.

5.2.2 Cultivation experiment

Four replicate plates per dilution and sampling site were incubated under hypoxic (2% O_2 [vol/vol], CO_2 (2% [vol/vol] and 96% N_2 [vol/vol]) atmosphere at room temperature in a cultivation system as illustrated in Figure 1. All cultures were maintained under low light conditions at room temperature (24 to 26 ^{o}C) for six weeks and the number of colonies then counted. Colonies used to determine colony counts were visible using a colony counter fitted

with 1.5X magnifying lens. Medium VL55 base contained 3.9 g 2-[*N*-morpholino]ethanesulfonic acid, 0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 0.4 mM $(NH_4)_2HPO_4$ and 2 mL of selenite/tungstate solution (TSCHECH; PFENNIG, 1984) per litre, and the pH was adjusted to 5.5 with a mixture of 200 mM NaOH plus 100 mM KOH. This medium base was autoclaved at 121 ^oC for 20 min and cooled to 56 ^oC. Ten millilitres of 5% (w/v) xylan from beechwood (Fluka), 2 mL of vitamin solution (see below) and 2 mL of trace element solution SL-10 (WIDDEL et al., 1983) with inclusion of aluminium potassium sulphate (0.01 g/L) were added per litre of medium base. The selenite/tungstate, vitamin and trace element solutions were sterilized by filtration. Vitamin solution contained (per litre of H_2O) 2 mg of (+)-biotin, 2 mg folic acid, 10 mg of pyridoxamine hydrochloride, 5 mg of thiamine chloride, 5 mg of riboflavin, 5 mg of nicotinic acid, 5 mg of hemicalcium D-(+)-pantothenate, 0.1 mg of cyanocobalamin, 5 mg of 4-aminobenzoate and 5 mg of DL-6,8-thioctic acid. Cycloheximide 1 µg/ml was added in the medium. This medium was used for enumeration and subcultivation.

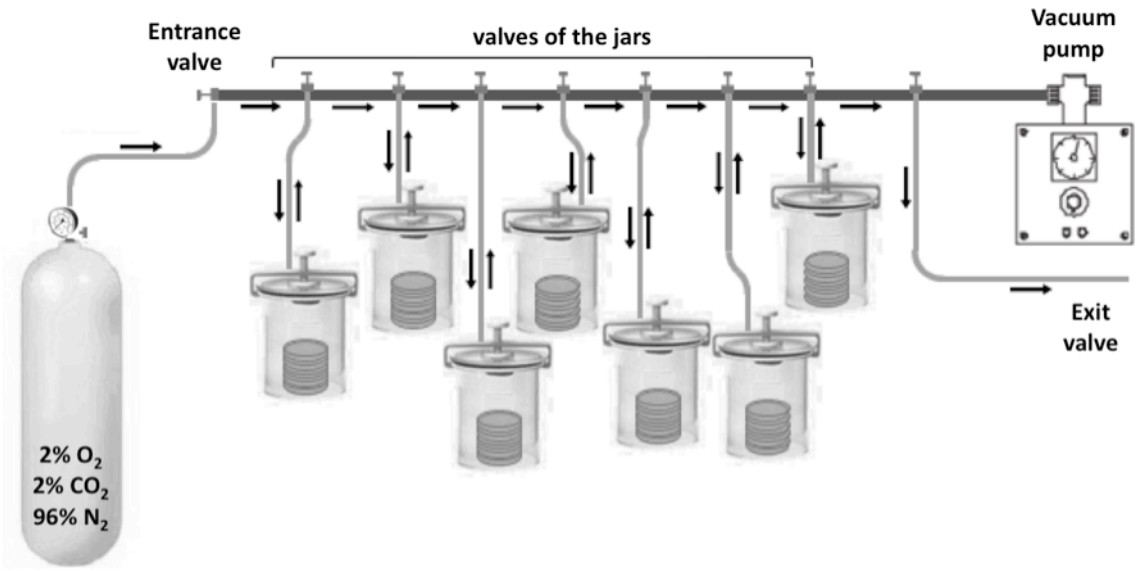

Figure 1 - Cartoon representing the cultivation system for soil bacteria as used in this study. The gas mixture (2% O_2 [vol/vol], 2% CO_2 [vol/vol] and 96% N_2 [vol/vol]) kept inside the cylinder was pulsed into the system daily after electric vacuum aspiration. Pressure control inside the anaerobic jars was based on a manometer located in the opening of the gas cylinder

5.2.3 PCR-screening for *Acidobacteria* in culture plates

Screening for *Acidobacteria* was performed after six weeks of incubation with group-specific primers. For picking isolated colonies, the plates were held on illumination with cool white light. All individual colonies or defined pools of the colonies were subjected to screening by the PCR after cells lysis in 50 µL of 1x Tris-EDTA (TE) buffer pH 8.0 (10 mM Tris Cl, 1 mM EDTA pH 8.0) in thermal cycler (Applied Biosystems, CA, USA) by 10 min at 96^{o}C. Each 25 µL of PCR reaction mixture contained approximately 50 ng of template DNA, 1X reaction buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, 0.25 mM concentration of each deoxynucleoside triphosphate, 0.2 µM concentration of each forward (Acid31F) (BARNS et al., 1999) and reverse (1492R) (LANE, 1991) primer, and 1.0 U of Taq DNA polymerase (Invitrogen). PCR mixtures were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, CA, USA) for the following amplification cycles: 3 min at 95 ^{o}C; 30 cycles of 1.5 min at 95 ^{o}C; 1 min at 52 ^{o}C; 1.5 min at 72 ^{o}C; followed by 10 min at 72 ^{o}C. A positive control reaction was performed using template DNA from *Acidobacterium capsulatum* (DSM 11244) in PCR.

PCR products were analyzed by electrophoresis of 5 µL samples of reaction mixtures on 1% agarose gels at 100 V in 0.5X Tris-borate-EDTA, visualized by UV illumination after staining with GelRed (Biotium Inc., Hayward, CA, USA), and photographed. Based on *A. capsulatum*-positive product, cell lysis material from acidobacterial isolated colonies were identified and used as template in PCR reaction with the universal bacterial primers FD1 and RD1 (WEISBURG et al., 1991). Each 25 µL reaction mixture contained approximately 50 ng of template DNA, 1 × PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, a 0.1 mM concentration of each deoxynucleoside triphosphate, a 0.2 µM concentration of each forward (F) and reverse (R) primer, and 1.25 U of Taq DNA polymerase (Invitrogen). The PCR mixtures were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) for the following amplification cycles: 96 °C for 4 min; 30 cycles consisting of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) after analysis by gel electrophoresis. The PCR products were ligated into a pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into competent *E. coli* DH5α cells. Four clones per acidobacterial detected colony were randomly selected and sequenced. Full sequencing for the 16S rRNA gene was performed for each clone using the primers 27F (5’

AGAGTTTGATCMTGGCTCAG 3'), 1492R (5' TACGGYTACCTTGTTACGACTT 3'), 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3'), the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3730xl DNA analyzer capillary sequencer (Applied Biosystems) (Macrogen Inc. Company, South Korea). Taxonomy was assigned to the sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4) (http://rdp.cme.msu.edu/). Two full sequences (1488 bp in size) for the 16S rRNA gene of acidobacterial identified colonies – PA (2.6) and SC-2 (3.2) – were deposited in the GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KC121328 and KC121329, respectively. In additional, two partial sequences (approximately 1000 bp in size) for the 16S rRNA gene of acidobacterial identified colonies – PA (1.5) and PA (2.1) – were deposited in the GenBank Database under accession numbers KC121330 and KC121331, respectively.

5.2.4 DNA Isolation, amplification and construction of 16S rRNA clone libraries

To obtain template DNA from the aggregate of colonies present on an isolation plate, the surface of the growth medium was flooded with 2 mL of bead solution from the Power Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), and then a sterile spreader was used to suspend as much colony material as possible. Colony material was pooled from isolation plates for each of six soil inoculum. The bead solution with suspended cells was transferred to a dry bead tube from the DNA kit, 50 µL of lysozyme solution (50 mg/ml; Sigma-Aldrich) was added, and the tube was incubated for 30 min in a 37 °C water bath. After incubation, DNA extraction was performed according to the manufacturer's protocol. 16S rRNA gene fragments were amplified using the PCR universal bacterial primers FD1 and RD1 (WEISBURG et al., 1991) as described before. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) after analysis by gel electrophoresis. The PCR products were ligated into a pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into competent *E. coli* DH5α cells. A total of 437 clones were randomly selected and sequenced. Sequencing was performed using the vector primer M13F (HUEY; HALL, 1989), the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3730xl DNA analyzer capillary sequencer (Applied Biosystems). Libraries were constructed for the DNA samples from isolation plates inoculated with PF soils (PF-1 GenBank accession nos. JX574664 - JX574723 and PF-2 GenBank accession nos.

JX574724 - JX574770), SC soils (SC-1 GenBank accession nos. JX574771 - JX574828 and SC-2 GenBank accession nos. JX574829 - JX574911), PA soil (GenBank accession nos. JX574912 - JX574988) and ADE soil (GenBank accession nos. JX574989 - JX575075).

5.2.5 Clustering clones into operational taxonomic units and phylogenetic analysis

The 16S rRNA gene sequences were edited using Phred/Phrap (EWING et al., 1998). Taxonomy was assigned to the sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4) (http://rdp.cme.msu.edu/). Sequences were grouped into operational units (OTUs) using MOTHUR software (SCHLOSS et al., 2009). An OTU was defined as a group of sequences that exhibit ≥ 97% similarity to each other, using the average neighbour algorithm. A representative sequence for each OTU was determined by MOTHUR software as the sequence that has the minimum distance from all other sequences within the same OTU. In addition, the representative sequences for each OTU were assigned to a phylogenetic tree containing mostly related nucleotide sequence available from assessed databases (GenBank and Ribosomal Database Project). The sequences were aligned using ClustalX2 (LARKIN et al., 2007) and further clustered by neighbor joining analysis on Kimura-2 parameters from a study conducted using MEGA version 4.0 (TAMURA et al., 2007), determining the preferred phylogenetic tree, supported by bootstrap values, based on an analysis of 1,000 subsamples.

5.3 Results and discussion

The recalcitrance of acidobacterial taxa members to growth on culture media has been extensively reported during the past decade. However, new cultivation strategies have been considered in combination to microbiology classical methods in order to improve the culturability of *Acidobacteria* on laboratory. Culturing techniques that have contributed to the successful isolation of *Acidobacteria* include: media with lowered pH (SAIT et al., 2006); increased headspace CO_2 concentrations (STEVENSON et al., 2004); substrate amendments (PANKRATOV et al., 2008); random selection of a wide variety of growth substrates (JOSEPH et al., 2003); use of a diffusion chamber (BOLLMANN et al., 2007) and extension of the incubation time (DAVIS et al., 2005). Nevertheless, *Acidobacteria* are a bacterial group

poorly represented in culture collection, without isolated member from Amazon soils. In the present work, we provide a first insight by culture-dependent approach about the cultivation in laboratory of acidobacterial members that inhabit different soils from Brazilian Amazon characterized as intact rainforest, deforested soils recently converted into cultivation and pasture, and Amazonian Dark Earth.

5.3.1 Colony counts of soil bacteria

In general, plates inoculated with 10^{-6} dilution of soil did not produced significant decrease in colony numbers per plate in comparison to plates inoculated with 10 times more soil (Figure 2). Colony forming units (CFU) counts ranged from a maximum of 4.3 x 10^7 g^{-1} of dry soil from ADE site to a minimum of 1.6 x 10^7 g^{-1} of dry soybean cropland soil (SC-2 field) (Figure 2). Isolation plates inoculated with soil from ADE site presented the higher number of bacterial colony, followed by plates inoculated with soils under-forest (PF-1 and PF-2), pasture and soybean cropland fields (SC-1 and SC-2) (Figure 2). In a temporal perspective, visible colonies appeared firstly in surface area of plates inoculated with soil from ADE site (after one week of incubation), secondly in plates inoculated with soils under-forest (after one week and half of incubation), and then in plates inoculated with soil from pasture and cropland fields (after two weeks of incubation). Three plates were unusable due to fungal contamination. This problem may be related to high richness of fungal populations in Amazon soils under-forest and in ADE sites (NAVARRETE et al., 2010; RUIVO et al., 2009).

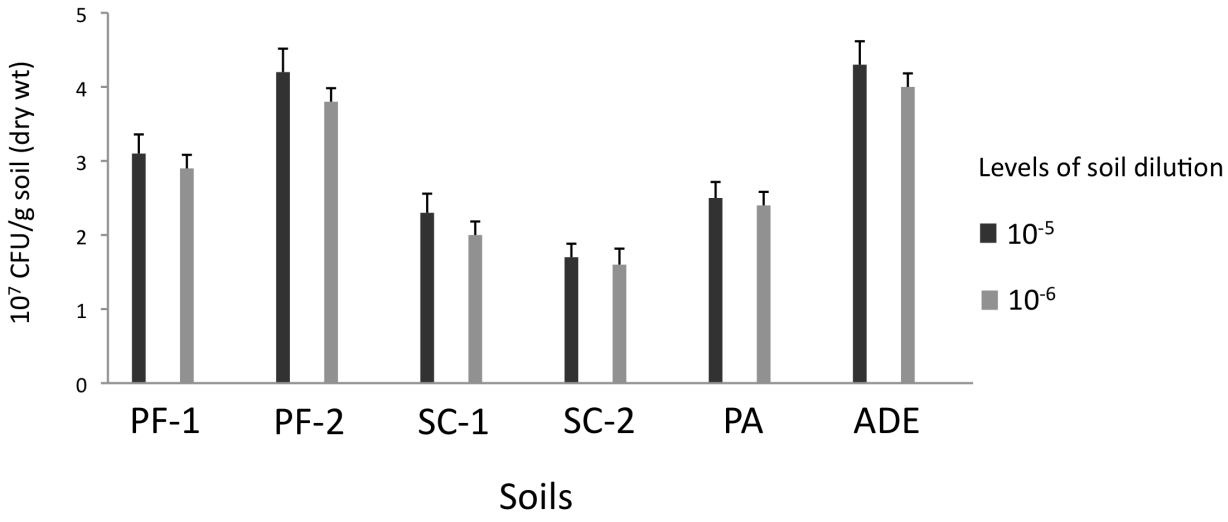

Figure 2 - Distribution of colony forming units (CFU) in the different soils used for the cultivation experiment. Each CFU count represents the mean of four plates at one dilution level, and was calculated based on the dry weight of the soil and dilution factors. The standard deviations are indicated as error bars

5.3.2 Molecular detection and identification of colonies belonging to the phylum *Acidobacteria*

A total of 456 colonies from the plates containing 10^{-5} and 10^{-6} dilutions of the soil as inoculum were picked and subjected to PCR-screening with primers targeting region of 16S rRNA-encoding gene specific to the phylum *Acidobacteria*. Only four acidobacterial colonies were detected from the total number of screened colonies by PCR. Three acidobacterial colonies were positively detected from plates inoculated with PA soil (colonies PA(1.5), PA(2.1) and PA(2.6)), and one acidobacterial colony was detected from a plate inoculated with SC-2 soil (colony SG-2(3.2)). Although the plates for the initial isolation experiment were incubated for six weeks, subcultures of the acidobacterial colonies PA(1.5) and PA(2.1) were able to hydrolyze the gellan gum within three weeks and made the plates unusable. These two acidobacterial colonies could not to be isolated. The PA(1.5) colony was identified as *Acidobacteria* subdivision 1 (100% identity with 16S rRNA gene sequences) and PA(2.1) colony as belonging to *Acidobacteria* subdivision 3 (100% identity with 16S rRNA gene sequences) (Figure 3). Based on full sequencing of the 16S rRNA gene the acidobacterial colonies PA(2.6) and SC-2(3.2) were identified as *Acidobacteria* subdivision 1 (100% identity with 16S rRNA gene sequences) (Figure 3). Both acidobacterial colonies PA(2.6) and

SC-2(3.2) were isolated and preserved by liofilization process and under mineral oil. All four acidobacterial colonies did not grow when inoculated in liquid VL55 medium. The colonies PA(2.6) and SC-2(3.2) were able to grow in solid medium under atmosphere unamended air. Subdivision 1 isolates (PA (2.6)) formed smooth and semi-transparent colonies, generally with an irregular margin at a later stage of development (> 2 months of incubation) (Figure 3).

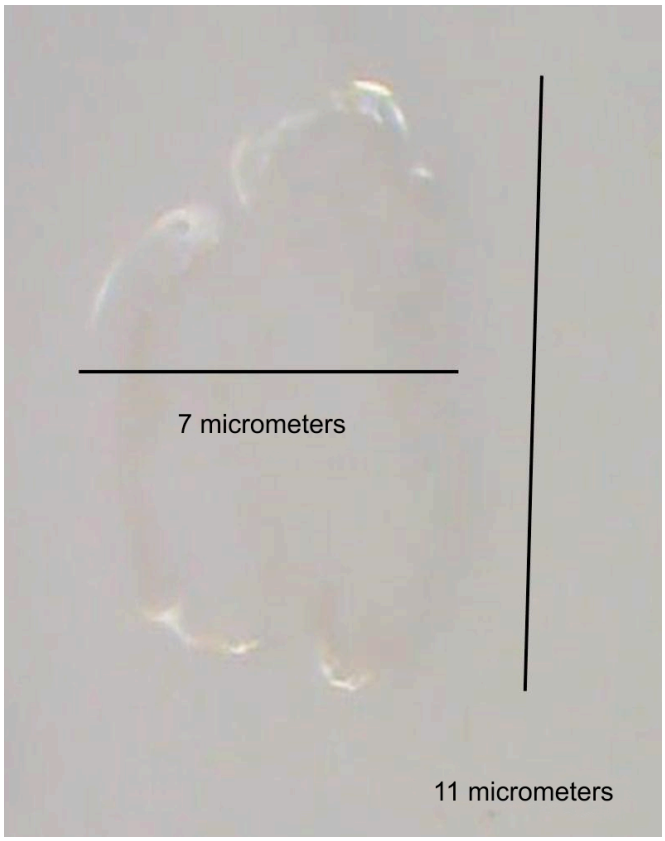

Figure 3 - Example of *Acidobacteria* colony recovered from pasture soil after 2 months of incubation on gas mixture (2% O2 [vol/vol], 2% CO2 [vol/vol] and 96% N2 [vol/vol]). The picture was taken from the upper of the plate with a 35x magnification using a Luxeo 4D Zoom Stereo Microscope with integrated camera (Labomed)

5.3.3 Culture-independent assessment based on 16S rRNA gene library

Obtaining visible colonies is a factor that may limit apparent culturability of some microorganisms. All colonies on a plate do not develop at the same rate, even in pure cultures (ISHIKURI; HATTORI, 1985; MOCHIZUKI; HATTORI, 1986). Some cells, after extraction from soil, develop into microcolonies of just a few cells (WINDING et al., 1994). It is not

clear if micro- or minicolonies would form visible colonies if incubated even long time, or if they have a self-limiting growth phenotype (DAVIS et al., 2011). However, in order to avoid that acidobacterial colonies could not be detected by picking isolated colonies, we used culture-independent assessment based on 16S rRNA gene clone library to searching for growth of *Acidobacteria* on surface area of the medium in the plate.

Of the bacterial 16S rRNA genes present in plates after six weeks of incubation of the Amazon soils from PF, SC, PA and ADE sites, 437 sequences were recovered. Taxonomy assignment showed that acidobacterial clones (≥ 97% identity with 16S rRNA gene sequences) were recovered only from plates inoculated with SC and PA soils (Table 1; Figure 3). Sequences belonging *Acidobacteria* subdivision 1 accounted for 10% and 23% of the total number of clones from plates inoculated with SC-2 and PA soils, respectively. One sequence belonging *Acidobacteria* subdivision 3 was recovered from plate inoculated with SC-1 soil (Table 1; Figure 3). This finding expands the result obtained with PCR-screening using specific-primers that showed detection of *Acidobacteria* subdivision 3 representative only from plate inoculated with PA soil.

Although comparative phylogenetic analysis of the DNA sequences of 16S rRNA genes has revealed that *Acidobacteria* subdivisions 1 and 3 are dominant in soil from the same sampling sites under-forest and cropland fields evaluated in this study (NAVARRETE et al., 2012), representatives of these group were recovered only from isolation plates inoculated with soybean cropland and pasture soils (Table 1; Figure 3). Eichorst et al. (2011) also detected members of subdivision 1 and 3 in the phylum *Acidobacteria* using molecular and cultivation-based approaches with agricultural and managed grassland soils in Michigan. The authors suggested that select members of these two acidobacterial subdivisions have the potential to play an active role in the degradation of plant polymers in bulk soil and utilize sugars from plant root exudates at various concentrations in the rhizosphere.

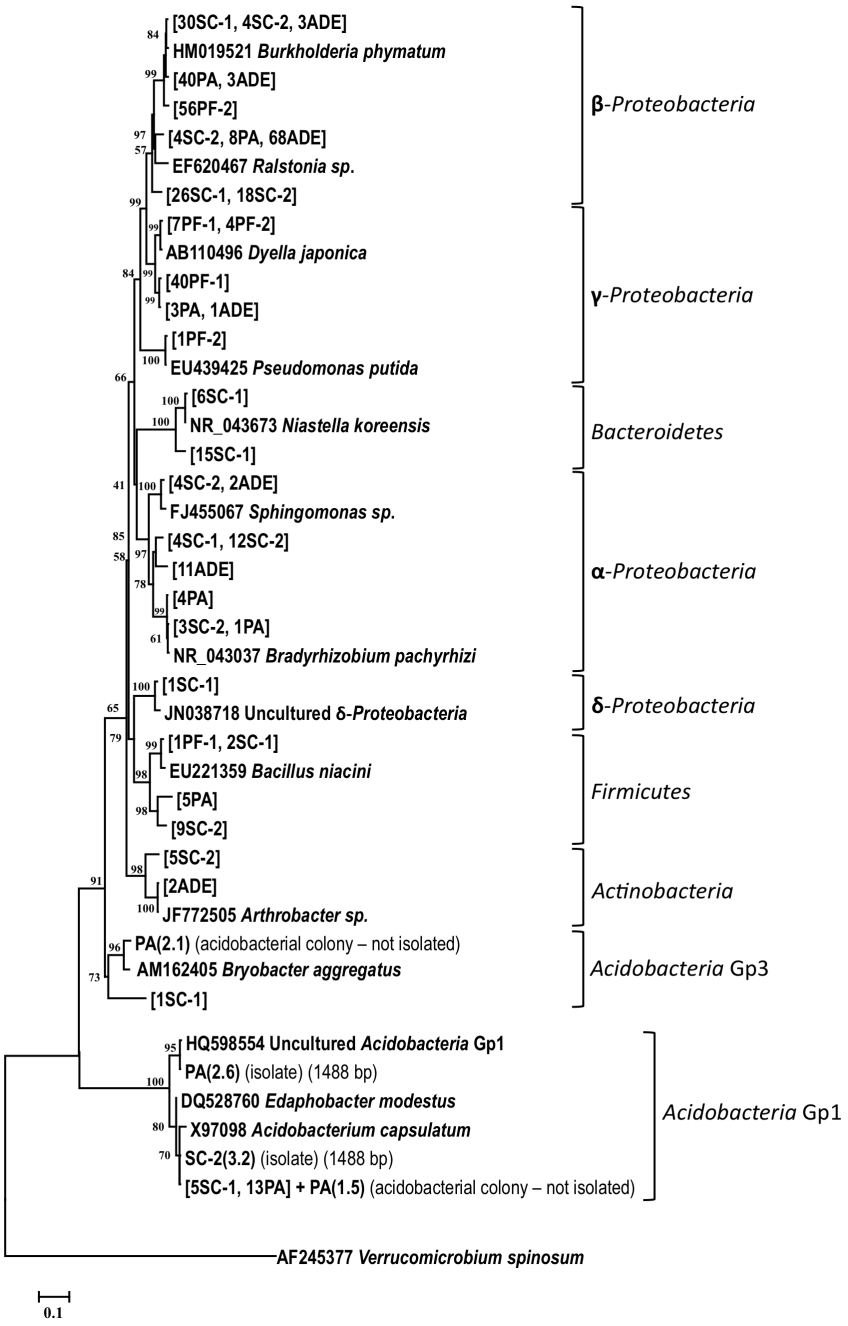

Figure 4 - Phylogenetic relationships based on partial 16S gene sequences of bacteria originating from isolation plates inoculated with Amazon soils from different origins (PF: primary forest areas, SC: soybean cropland fields, PA: pasture and ADE: Amazonian Dark Earth sites) with their best matches in the databases searched (GenBank and Ribossomal Database Project). A bootstrap analysis was performed with 1,000 repetitions, and values indicate the percentage of clustering matches. The scale bar at the bottom of the figure displays the number of differences in base composition among sequences. Values and abbreviations in brackets indicate the number of clones in each OTU (3% cutoff point of dissimilarity) and their origins, respectively. Soil samples (PF and SC) were collected in sites located at Porto dos Gaúchos municipality (1) and the Ipiranga do Norte municipality (2)

Table 1 - Taxa and numbers of clones derived from isolation plates inoculated with soil collected from primary forest areas, soybean cropland fields, pasture and Amazonian Dark Earth sites

Phylum	Class	Order	Clone libraries					
			PF-1	PF-2	SC-1*	SC-2*	PA*	ADE
Acidobacteria	Subdivision 1		0	0	0	6	19	0
	Subdivision 3		0	0	1	0	0	0
Actinobacteria	*Actinobacteria*	*Actinomycetales*	0	0	0	5	0	2
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	0	0	21	0	0	0
Firmicutes	*Bacilli*	*Bacillales*	1	0	2	9	5	0
Proteobacteria	**α**-*Proteobacteria*	*Rhizobiales*	0	0	4	7	4	10
		Sphingomonadales	0	0	0	4	0	1
		unclassified	0	0	0	1	0	0
	β-*Proteobacteria*	*Burkholderiales*	0	56	56	28	48	74
		Rhodocyclales	0	0	2	0	0	0
		unclassified	0	1	0	0	0	0
	δ-*Proteobacteria*	*Myxococcales*	0	0	1	0	0	0
	γ-*Proteobacteria*	*Xanthomonadales*	47	3	0	0	3	0
		Pseudomonadales	0	2	0	0	0	0
		Enterobacteriales	0	0	0	0	0	1
	unclassified		0	0	2	2	1	1
unclassified			2	0	1	1	1	2

PF, soil from primary forest; SC, soil from soybean cropland fields; PA, soil from pasture; ADE, Amazonian Dark Earth. Soil samples (PF and SC) were collected in sites located at Porto dos Gaúchos municipality (1) and the Ipiranga do Norte municipality (2).

*Libraries from which derived acidobacterial colonies. Acidobacterial isolates were recovered from isolation lates inoculated with SC-2 and PA soils.

Some growth conditions have been considered in cultivation experiment in order to bring a better representation of acidobacterial diversity into laboratory culture. Sait et al. (2006) identified moderately acidic pH values as an important factor in their success in isolating many members of subdivision 1 of the phylum *Acidobacteria*. This fits with the abundance of members of this group in different soils, in which they form relatively larger parts of the total community with decreasing soil pH (SAIT et al., 2006). Pure cultures also display optima for growth at moderately acidic pH values of 4.5 to 6 (EICHORST et al., 2007). Stevenson et al. (2004) found that increased CO_2 partial pressures, mimicking those found in soils (RUSSELL, 1950), increased the culturability of *Acidobacteria* subdivision 1, but Sait et al. (2006) and Eichorst et al. (2007) later showed that this was an effect on the medium pH. Elevated CO_2 partial pressure could be particularly significant for any potentially autotrophic groups, and different subsets of the soil community may have been cultured.

Clones in library from plates inoculated with PF-1 soil consisted majoritarily of *Proteobacteria* (order *Xanthomonadales*). Representatives of phylum *Proteobacteria* (order *Burkholderiales*) were more present in PF-2 library. Clones from plates inoculated with SC-1 and SC-2 soils were commonly related to the phyla *Proteobacteria* (order *Burkholderiales* and *Rhizobiales*) and *Firmicutes* (order *Bacillales*). However, sequences belonging to *Bacteroidetes* (order *Sphingobacteriales*) were recovered only from clones in SC-1 library. *Actinobacteria* (order *Actinomycetales*) and *Acidobacteria* subdivision 1 were belonged to sequences from clones in SC-2 library, but not in SC-1 library. Clones derived from isolation plates inoculated with PA soils were mostly affiliated with *Proteobacteria* (order *Burkholderiales*), followed by *Acidobacteria* subdivision 1. In isolation plates inoculated with ADE soil the clones were mostly related to the phylum *Proteobacteria* (orders *Burkholderiales* and *Rhizobiales*) (Table 1; Figure 3). Furthermore, taxonomically unclassified bacteria were found in all libraries, except in PF-2 library.

Considering the differential occurrence of *Acidobacteria* on isolation plates in contrast to the growth of other bacterial groups (Table 1), it is interesting to note that based on the strategy of the cultivation procedure considered in this study, acidobacterial colonies were detected in plates where was accounted lower number of bacterial colonies and longer time to start visible bacterial growth in surface area of the plate (Table 1; Figure 2). The slow-growing microorganisms may be inhibited by products from more rapidly growing colonies (JANSSEN, 2008; VARTOUKIAN et al., 2010). These are some of the reasons that widely

used cultivation methods fail to culture the full extent of the phylogenetic diversity of bacteria present in environmental samples (HUGENHOLTZ et al., 1998). This has been particularly apparent in attempts to culture soil bacteria, where both the number (CONN, 1918) and diversity (JANSSEN, 2006) are greatly underestimated by current cultivation-based techniques.

5.4 Conclusion

In conclusion, this work reports the recovery and detection of hitherto-uncultured representatives of *Acidobacteria* subdivisions 1 and 3 on VL55 growth medium from Amazon soils under soybean cropland and pasture. Our results indicate that there is a culturable diversity of *Acidobacteria* from Amazon soils that have generally eluded from laboratory study by cultivation methods, but that can be recovered and detected on laboratory medium when working at both cultivation and culture-independent approaches.

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6 GENERAL CONCLUSIONS

In section 2 of this thesis the research objectives were formulated and described. In section 3 the study entitled "Amazonian deforestation alters bacterial networks and verrucomicrobial communities in soil" described an unprecedented ecological approach that couples bacterial 16S ribosomal DNA pyrotag, shotgun metagenomics and measurements of environmental parameters from soil sampled in three replicate sites of mature forest and adjacent deforested site after 2-4 months of forest clearing and burning in the Brazilian Amazon so to address the following research questions:

Which bacterial taxonomic groups are responsive to deforestation effects in Amazon forest soils?

Is the complexity of association networks of bacteria to bacteria and to soil characteristics and function to function to soil affected by clearing and burning of intact mature Amazon rainforest?

As a response to these key questions the thesis has presented that deforestation led to a consistent decline in the abundance of *Verrucomicrobia* and alterations in verrucomicrobial community structure. Furthermore, deforestation of Amazon forest soils quickly leads to simplification of the association networks among different bacterial taxonomic groups, and in order to adapt to this condition function-based associations network were enhanced, indicating a higher degree of risk spreading for the maintenance of soil functioning.

The scope contemplated by study presented in section 4 sought to obtain a better insight into the ecological characteristics of *Acidobacteria* in Amazon soils and in soils recently converted into cultivation. In summary, acidobacterial community in bulk soil samples from cropland fields and adjacent native forests as well as in the rhizosphere of soybean was assessed by molecular tools i.e. quantitative real-time PCR and pyrosequencing spanning the V4 region of the 16S rRNA gene. *Acidobacteria* subgroups 4, 6 and 7 revealed preponderant responses to abiotic soil factors, which were affected by the changes in soil uses from native forests into soybean production fields. Subgroup-level relative abundance of *Acidobacteria* correlated with soil factors linked to soil acidity, such as pH, Al, Al saturation and base saturation. Interestingly, other soil factors also contribute to explain the variability in acidobacterial relative abundance, including Ca, Mg, Zn, P, Fe, Mn and B. These findings can

help to reveal what drives acidobacterial population changes in Amazon soils recently converted into cultivation and open the possibilities to explore acidobacterial subgroups as early-warning bio-indicators of agricultural soil management effects in the wide Amazon area.

Section 5 presented several important considerations regarding to combination of cultivation and molecular approaches to search for growth of hitherto-uncultured *Acidobacteria* concomitantly to other bacterial groups inhabiting Amazon soils. The presented results reported the recovery and detection of hitherto-uncultured representatives of *Acidobacteria* subdivisions 1 and 3 on VL55 growth medium from Amazon soils under soybean cropland and pasture, and indicated that there is a culturable diversity of *Acidobacteria* from Amazon soils that have generally eluded from laboratory study by cultivation methods, but that can be recovered and detected on laboratory medium when working at both cultivation and culture-independent approaches.

In general, the contribution of the research work presented in this thesis is a better understanding about the consequences of clearing and burning of Amazon rainforest on soil-borne bacterial communities and soil functioning; the insight about the possibility to explore acidobacterial subgroups as early-warning bio-indicators of agricultural soil management effects in the Amazon area; and validation of a cultivation and detection procedure of hitherto-uncultured *Acidobacteria* from Amazon soils. These findings are especially imperative in the current time for the Amazon rainforest because this ecosystem is under great threat in consequence of an increasing demand for grain, fibers and biofuel.

APPENDICES

APPENDIX A: Supplementary Tables

Supplementary Table 1 - Characteristics of the three real replications of mature forest and adjacent deforested sites in the Amazon rainforest and sampling points under forest and deforested sites located at Southeastern Brazilian Amazon. Section 3 - Amazonian deforestation alters bacterial networks and verrucomicrobial communities in soil

Area	Coverage	Sampling points	Latitude/longitude	Sampling and field notes
1	forest	FS1.A	15°11'45''S/54°03'31"W	High density forest
		FS1.B	15°11'43''S/54°03'31"W	
		FS1.C	15°11'45''S/54°03'29"W	
		FS1.D	15°11'47''S/54°03'31"W	
		FS1.E	15°11'45''S/54°03'33"W	
	deforested	DS1.A	15°09'45''S/54°03'31"W	4 months after slash-and-burn. Soil non-recolonised by plants. Little burned plant residues incorporated (trunks and branches of trees)
		DS1.B	15°09'43''S/54°03'31"W	
		DS1.C	15°09'45''S/54°03'29"W	
		DS1.D	15°09'47''S/54°03'31"W	
		DS1.E	15°09'45''S/54°03'33"W	
2	forest	FS2.A	14°21'38''S/54°21'27"W	High density forest
		FS2.B	14°21'36''S/54°21'27"W	
		FS2.C	14°21'38''S/54°21'25"W	
		FS2.D	14°21'40''S/54°21'27"W	
		FS2.E	14°21'38''S/54°21'29"W	
	deforested	DS2.A	14°19'38''S/54°22'27"W	4 months after slash-and-burn. Soil non-recolonised by plants. Little burned plant residues incorporated (trunks and branches of trees)
		DS2.B	14°19'36''S/54°22'27"W	
		DS2.C	14°19'38''S/54°22'25"W	
		DS2.D	14°19'40''S/54°22'27"W	
		DS2.E	14°19'38''S/54°22'29"W	
3	forest	FS3.A	13°21'57''S/54°54'24"W	High density forest
		FS3.B	13°21'55''S/54°54'24"W	
		FS3.C	13°21'57''S/54°54'22"W	
		FS3.D	13°21'59''S/54°54'24"W	
		FS3.E	13°21'57''S/54°54'26"W	
	deforested	DS3.A	13°22'57''S/54°56'24"W	2 months after slash-and-burn. Soil non-recolonised by plants. Little burned plant residues incorporated (trunks and branches of trees)
		DS3.B	13°22'55''S/54°56'24"W	
		DS3.C	13°22'57''S/54°56'22"W	
		DS3.D	13°22'59''S/54°56'24"W	
		DS3.E	13°22'57''S/54°56'26"W	

Supplementary Table 1 - List of public access codes of the clean acidobacterial sequences uploaded to the metagenomic RAST (MG-RAST) server. Section 4 - Acidobacterial community responses to agriculture management of amazon forest soils

ID samples	MG-RAST accession numbers
BS-F1	4497244.3
BS-F2	4497245.3
BS-F3	4497246.3
BS-F4	4497247.3
BS-C1	4497240.3
BS-C2	4497241.3
BS-C3	4497242.3
BS-C4	4497243.3
SR-I	4497248.3
SR-II	4497249.3
SR-III	4497250.3
SR-IV	4497251.3

Supplementary Table 2 - Soil chemical factors of the 0- to 20-cm topsoil layer at different sampling sites. Section 4 - Acidobacterial community responses to agriculture management of Amazon forest soils

Soil factors	Bulk Soil - Forest (BS-F)				Bulk Soil - Cropland (BS-C)			
	BS-F1	BS-F2	BS-F3	BS-F4	BS-C1	BS-C2	BS-C3	BS-C4
pH	3.7[(1)]a[(2)] ± 0.1	4.1b ± 0.1	4.6A ± 0.2	4.7A ± 0.2	4.1a ± 0.3	5.7b ± 0.2	5.0A ± 0.2	5.1A ± 0.2
OM	37.5a ± 3.8	36.6a ± 4.1	32.0A ± 4.6	31.8A ± 1.7	25.8a ± 3.8	28.0a ± 3.6	35.2A ± 2.4	27.2B ± 1.5
Al	17.0a ± 1.8	15.0a ± 2.5	12.0A ± 1.2	12.4A ± 1.1	9.8a ± 3.6	0.2b ± 0.4	2.0A ± 0.7	2.8A ± 1.3
H+Al	71.5a ± 5.4	65.6a ± 8.8	67.4A ± 5.4	61.4A ± 4.7	42.6a ± 13.7	27.0a ± 8.5	50.4A ± 8.6	34.8A ± 13.8
m	75.7a ± 2.5	80.2a ± 6.4	82.4A ± 1.3	78.2A ± 10.1	52.4a ± 14.5	6.0b ± 1.3	8.0A ± 4.41	15.0A ± 7.3
P	5.2a ± 1.0	4.4a ± 0.5	4.4A ± 0.5	4.8A ± 0.8	11.2a ± 18.8	7.4a ± 3.4	33.6A ± 20.9	13.2A ± 5.2
K	1.0a ± 0.1	0.5b ± 0.1	0.5A ± 0.05	0.34B ± 0.05	0.8a ± 0.3	0.6a ± 0.3	1.7A ± 0.7	0.6B ± 0.2
Ca	2.5a ± 0.6	2.0a ± 0.7	1.0A ± 0	2.4A ± 2.6	5.2a ± 1.8	27.6b ± 9.4	13.6A ± 3.0	10.2A ± 2.2
Mg	2.0a ± 0	1.2b ± 0.4	1.0A ± 0	1.0A ± 0	2.8a ± 3.6	22.2b ± 10.0	9.0A ± 2.1	5.6A ± 1.3
SB	5.5a ± 0.7	3.7a ± 1.1	2.5A ± 0.05	3.7A ± 2.6	8.8a ± 3.1	50.4b ± 19.2	24.3A ± 5.4	16.4A ± 3.6
CEC	77.0a ± 5.4	69.3a ± 8.2	70.0A ± 5.5	65.1A ± 5.6	51.4a ± 13.9	77.4a ± 13.3	74.7A ± 12.0	51.2A ± 15.0
V	7.0a ± 1.4	5.2a ± 2.3	3.8A ± 0.4	5.8A ± 3.5	18.2a ± 7.7	63.6b ± 14.8	32.4A ± 4.6	34.8A ± 12.5
B	0.3a ± 0.05	0.3a ± 0.03	0.2A ± 0.01	0.2A ± 0.01	0.2a ± 0.03	0.2a ± 0.02	0.1A ± 0.04	0.2A ± 0.03
Cu	0.3a ± 0.08	1.3b ± 0.8	0.2A ± 0	0.2A ± 0.07	0.3a ± 0	0.4b ± 0.07	0.3A ± 0.06	0.3A ± 0.05
Fe	308.0a ± 44.8	293.4a ± 93.5	85.4A ± 8.9	120.0A ± 32.3	93.2a ± 49.5	85.0a ± 22.4	47.6A ± 6.2	62.2A ± 8.8
Mn	2.0a ± 1.1	2.0a ± 0.8	1.0A ± 0.3	1.7A ± 0.4	1.6a ± 0.5	1.0a ± 0.4	0.5A ± 0.2	0.7A ± 0.1
Zn	0.2a ± 0	1.1a ± 1.0	0.2A ± 0.1	0.3A ± 0.1	0.9a ± 1.3	0.6a ± 0.08	2.6A ± 2.1	0.9A ± 0.2

Ca, Mg, K, Al, potential acidity (H+Al), sum of base (SB) are expressed in $mmol_c\ kg^{-1}$; organic matter (OM) is expressed in $g\ kg^{-1}$; P is expressed in $mg\ kg^{-1}$; B, Fe, Mn, Zn, K, Cu and cation exchange capacity in pH 7 (CEC) are expressed in $mg\ dm^{-3}$. m – Al saturation index. V – base saturation index.

(1) The values are averages based on quintuplicate sampling points in each site. Standard deviations are shown in the table.

(2) Values with the same lower or upper-case letters were not significantly different ($P<0.05$) based on upon a Tukey's HSD test. Tukey's test was performed separately for field location and sampling period (samples 1-2 and 3-4).

Supplementary Table 3 - Coefficient of determination (R^2) values for significant correlations between relative abundance of acidobacterial subgroups and soil factors. Section 4 - Acidobacterial community responses to agriculture management of amazon forest soils

	Acidobacteria subgroups							
Soil factors	1	2	3	4	6	7	10	13
pH			0.50099	0.64737	0.66585	0.71876	0.50539	
OM								
Al				0.84756	0.90746	0.84761	0.65205	
H+Al	0.45953			0.78651	0.68081	0.5142	0.78671	
m	0.5437			0.87159	0.94669	0.88347	0.52355	
P								0.64425
K								
Ca				0.58893	0.77914	0.96934		
Mg					0.67702	0.91744		
SB				0.5142	0.68081	0.78651		
CEC								
V				0.71532	0.80577	0.95848		
B					0.68081			0.65166
Fe		0.43391						
Mn		0.43391		0.78651	0.68081			
Zn								0.65166
Cu								

Values obtained based on linear function.

FEMS MICROBIOLOGY ECOLOGY

APPENDIX B - Published article

RESEARCH ARTICLE

Acidobacterial community responses to agricultural management of soybean in Amazon forest soils

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soil microbiology; soil factors; land-use changes; tropical rainforest; 16S rRNA gene.

Abstract

This study focused on the impact of land-use changes and agricultural management of soybean in Amazon forest soils on the abundance and composition of the acidobacterial community. Quantitative real-time PCR (q-PCR) assays and pyrosequencing of 16S rRNA gene were applied to study the acidobacterial community in bulk soil samples from soybean croplands and adjacent native forests, and mesocosm soil samples from soybean rhizosphere. Based on qPCR measurements, *Acidobacteria* accounted for 23% in forest soils, 18% in cropland soils, and 14% in soybean rhizosphere of the total bacterial signals. From the 16S rRNA gene sequences of *Bacteria* domain, the phylum *Acidobacteria* represented 28% of the sequences from forest soils, 16% from cropland soils, and 17% from soybean rhizosphere. *Acidobacteria* subgroups 1–8, 10, 11, 13, 17, 18, 22, and 25 were detected with subgroup 1 as dominant among them. Subgroups 4, 6, and 7 were significantly higher in cropland soils than in forest soils, which subgroups responded to decrease in soil aluminum. Subgroups 6 and 7 responded to high content of soil Ca, Mg, Mn, and B. These results showed a differential response of the *Acidobacteria* subgroups to abiotic soil factors, and open the possibilities to explore acidobacterial subgroups as early-warning bioindicators of agricultural soil management effects in the Amazon area.

Introduction

Soil bacterial communities in the Amazon area have been analyzed in different types of soils (Borneman & Triplett, 1997; Kim *et al.*, 2007; Cenciani *et al.*, 2009; Jesus *et al.*, 2009; O'Neill *et al.*, 2009; Navarrete *et al.*, 2010). Based on these studies, the bacterial community composition was revealed in soils from different Amazon regions. The *Acidobacteria* phylum has been described as dominant in soils from Western Amazon (Kim *et al.*, 2007; Jesus *et al.*, 2009) and Central Amazon (Navarrete *et al.*, 2010). However, the role of this dominant group in the bacterial community of Amazon soils is largely unknown.

Acidobacteria have consistently been detected in many different habitats around the globe by 16S rRNA gene–based molecular surveys, including soil and rhizosphere niches (Chow *et al.*, 2002; Kuske *et al.*, 2002; Gremion *et al.*, 2003; Quaiser *et al.*, 2003; Fierer *et al.*, 2005; Stafford *et al.*, 2005; Janssen, 2006; Sanguin *et al.*, 2006; De Cárcer *et al.*, 2007; Singh *et al.*, 2007; DeAngelis *et al.*, 2009; Kielak *et al.*, 2009). These observations have revealed that *Acidobacteria* are ubiquitous and among the most abundant bacteria phylum in soil. In spite of their high abundance, little information is available on their ecology, which is mainly due to the lack of culturable representatives in bacterial collections (Kishimoto *et al.*, 1991; Liesack *et al.*, 1994; Coates *et al.*, 1999; Bryant *et al.*, 2007; Eichorst *et al.*, 2007, 2011; Fukunaga *et al.*, 2008; Koch *et al.*, 2008; Lee *et al.*, 2008; Nunes da Rocha *et al.*, 2009; Ward *et al.*, 2009; Kulichevskaya *et al.*, 2010; Pankratov & Dedysh, 2010; Männistö *et al.*, 2011; Pankratov *et al.*, 2011).

Land-use changes is one of the greatest threats to biodiversity worldwide, and one of the most devastating

land-use changes, especially in the tropics, is the conversion of intact forests into cultivation fields (Morton *et al.*, 2006). The Amazon is a vast, yet vulnerable, hotspot of biodiversity, and the maintenance of soil fertility is critical to sustaining these diverse ecosystems. With this in mind, increased attention has recently been paid to belowground biodiversity in the Amazonian region, and a number of cultivation-independent studies have sought to assess the impact of land-use changes on microbial communities resident to Amazon soils (Borneman & Triplett, 1997; Cenciani *et al.*, 2009; Jesus *et al.*, 2009; Navarrete *et al.*, 2010, 2011). However, despite this increased appreciation of belowground microbial diversity in the Amazonian region, little is still known about bacterial taxa responses to alterations in soil chemical properties and fertility in consequence to deforestation and agricultural management of Amazon forest soils.

Because of the substantial effects that land-use changes may have on the chemical and physical characteristics of former tropical forest soils, and the high abundance and presumed importance of *Acidobacteria* for the functioning of soil systems, we would like to obtain better insight into the ecological characteristics of *Acidobacteria* in Amazon soils and in soils recently converted into cultivation. For this purpose, we applied quantitative real-time PCR and pyrosequencing spanning the V4 region of the 16S rRNA gene to analyze the abundance and the composition of the acidobacterial community inhabiting bulk soil from soybean croplands and adjacent forests collected in an agricultural zone located in the Southeastern Brazilian Amazon as well as soybean rhizosphere soil from mesocosm experiment. The relative abundances of *Acidobacteria* at the phylum and subgroup taxonomic levels in soil samples were correlated with soil factors in order to explore responses to alterations in soil characteristics due to land-use changes and agriculture management of Amazon forest soils.

Materials and methods

Site description and soil sampling

Bulk soil samples were collected in two different field locations in the Southeastern Brazilian Amazon, in the state of Mato Grosso, Brazil, in the Porto dos Gaúchos municipality (−15°13′39″ S and −54°04′31″ W) and the Ipiranga do Norte municipality (−13°21′57″ S and −54° 54′24″ W) (Fig. 1). Oxisol is the predominant soil order in the sampling sites (Secretaria de Estado de Planejamento e Coordenação Geral, 2001), and the climate in the region is classified as Am (Koppen's classification), with annual average temperature of 28 °C and average precipitation of 2000 mm.

The field locations were considered replicates and the sampling sites were selected according to the vegetation cover, soil use, and management practices. In the Porto dos Gaúchos municipality, areas covered with native tropical rainforest were cleared in 2008 and subsequently converted into agricultural land. Since 2004, forest conversion to agricultural use occurred in areas located in Ipiranga do Norte municipality. In both field locations, forest conversion to agricultural use followed annually the rotational production order: millet, soybean, maize, under no-tillage. After deforestation, fertilizers, pesticides, and a liming treatment were applied to the cropland fields of both locations. The cropland fields received different amounts of lime to increase soil pH to 5 and 6.

Bulk soil samples were collected from soybean production fields before sowing the seeds (October 2009) and after soybean (*Glycine max* [L.] Merrill cultivar M-SOY 8866) harvest (April 2010) in order to consider an expected variation in soil characteristics during the soybean cultivation (Fig. 1). Soil samples were also collected at the same time from adjacent forests to represent the native soil–plant conditions (Fig. 1). At each sampling site, the soil samples were collected from five points. One central sampling point and other four sampling points (at least 50 m apart from the central point) directed toward the north, south, east, and west of the central point. Soil samples were taken from the 0- to 20-cm topsoil layer (tilled zone). First, the litter layer was removed, and then, the soil sample was collected using a 5-cm-diameter aseptic cylindrical core. A total of 40 bulk soil samples were collected in field (2 field locations × 2 sampling sites per field location × 2 sampling periods × 5 sampling points per site). Samples were transported to the laboratory under ice and stored at −20 °C until processing within 72 h after sampling.

Abiotic soil factors

For soil physicochemical analysis, soil sample consisted of a composite sample for each sampling site and period by mixing five subsamples collected from the sampling points. From each composite sample, a subsample was removed, air-dried, and passed through a 2-mm mesh sieve and then analyzed according to Empresa Brasileira de Pesquisa Agropecuária (1997) at the Department of Soil Science at the University of São Paulo (ESALQ-USP) for the soil fertility properties. Soil pH was measured in a 1 : 2.5 soil/water suspension. Exchangeable Al, Ca, and Mg were extracted with KCl 1 M. Calcium and Mg were determined by atomic absorption spectrometry and Al by acid–base titration. Phosphorous and K were extracted by ion-exchange resin. Potential acidity (H + Al) was estimated by an equation based on the pH determined in

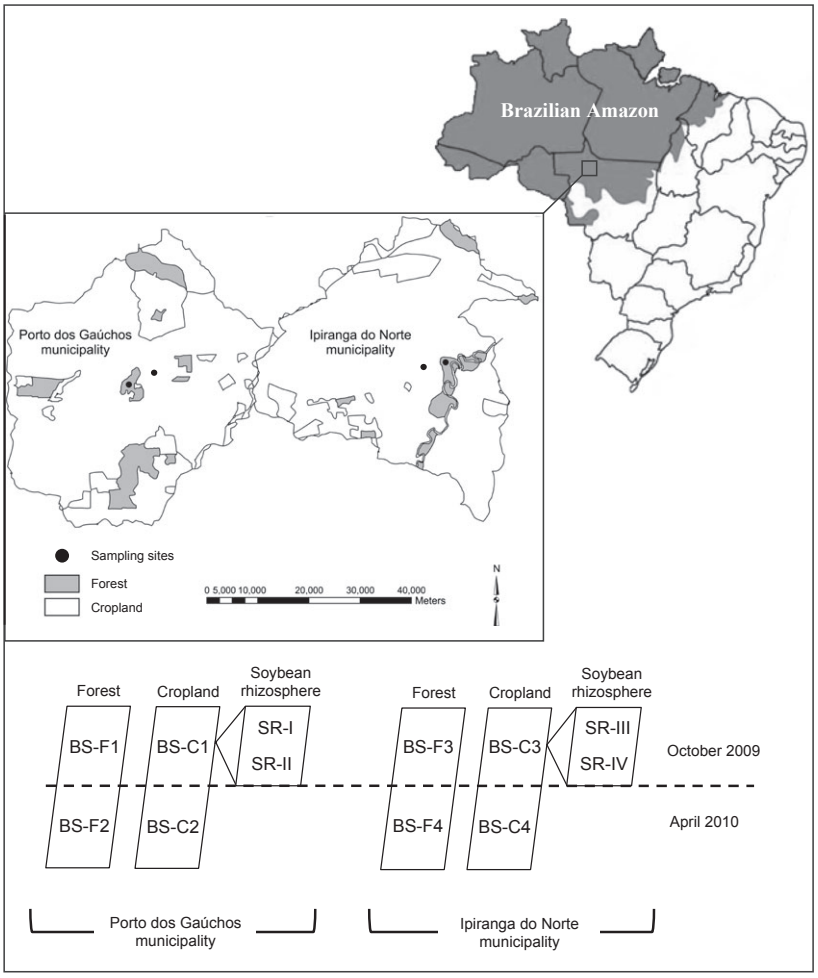

Fig. 1. Location of the sampling sites in the Southeastern Brazilian Amazon and time-location scheme for soil sampling (bulk soil from field and soybean rhizosphere from greenhouse mesocosm experiment). Sampling points were located under cropland and adjacent forest in both the Porto dos Gaúchos municipality (−15°13′39″ S and −54°04′31″ W) and the Ipiranga do Norte municipality (−13°21′57″ S and −54°54′24″ W), state of Mato Grosso, Brazil. Bulk soil sampling was performed before sowing soybean seeds (October 2009) and after soybean harvest (April 2010) in cropland sites. Concomitantly, bulk soil samples were taken in adjacent forests. In the time-location scheme for soil sampling, codes indicate the name attributed to the pooled DNA samples used for pyrosequencing analysis (BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere). The roman numerals I, II, III, and IV indicate the replication of greenhouse mesocosm experiment.

SMP buffer solution (pH SMP). Available micronutrients (Fe, Mn, Zn, and Cu) were extracted by Mehlich 1 and determined by atomic absorption spectrometry. Boron was extracted with hot water and determined by spectrophotometry with azomethine-H at 420 nm. Some of the results allowed the calculation of other parameters such as exchangeable bases (SB), the sum of Ca, Mg, and K; cation exchange capacity (CEC), the sum of Ca, Mg, K, Al, and H; base saturation (V), the percentual relation between SB and CEC; and Al saturation (m%), the percentual relation between exchangeable Al and CEC. Soil texture was determined using Bouyoucos densimeter after shaking the soil vigorously with NaOH 1 M as dispersant.

Statistical analysis for soil factors

Analyses of similarity (ANOSIM) of soil physicochemical properties were performed considering the differences in cropland and forest soils. Distance matrix (Euclidean metric) was constructed with nontransformed data. ANOSIM was carried out using PRIMER SIX (version 6.1.5; Primer-E Ltd, Plymouth, UK). Correlation analysis was performed in R (version 2.6.0; The R Foundation for Statistical Computing). Spearman's rank coefficients were calculated to investigate the correlation between the soil factors.

Soybean rhizosphere soil

In order to consider the abundance and composition of acidobacterial community in the soybean rhizosphere niche, normalizing the influence of environmental parameters (such as moisture regime and temperature) on the growth conditions for the plants, soybean plants were grown in mesocosms in the greenhouse at CENA-USP, Piracicaba, São Paulo. Soil samples were taken at the same five sampling points used for bulk soil collecting in soybean production fields before sowing the seeds. Each soil sample consisted of a composite sample by mixing five subsamples collected from the 0- to 20-cm topsoil layer. Twenty mesocosms in ceramic pots (30 cm high × 20 cm diameter) were filled with the soils.

Duplicate pots were filled with 8 kg of soil and disposed over a bottom of 5-cm layer filled with washed stones. Seeds of the same soybean cultivar (M-SOY 8866) used in field were germinated in the mesocosms at a regime of 12/12-h light/dark cycle and average temperatures of 28 °C at day and 19 °C at night. The moisture content of the pots was regularly adjusted by an external visual controller installed in each pot. The temperature and moisture regimes were chosen close to natural conditions to create optimal growth conditions for the plants. The plants were grown from November 2009 until January 2010 (84 days). A total of 20 mesocosm samples from soybean rhizosphere soil were taken in late January, prior to plant maturity (soil from 2 soybean production fields × 5 sampling points per field × 2 ceramic pots per sampling point). Roots and associated soil were transported to the laboratory on ice and then processed to obtain the rhizosphere soil. The roots were shaken to remove the loose soil, and the tightly attached soil including small aggregates (< 0.5 cm) was used for DNA extraction.

Isolation of DNA from soil and quantitative real-time PCR assay

DNA was extracted from 250 mg of bulk soil samples from croplands and adjacent native forests, and mesocosm samples from soybean rhizosphere soil using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. DNA was extracted in triplicate for each soil sample and stored at −20 °C until use. Quantitative real-time PCR (qPCR) using the 16S rRNA gene as biomarker was performed to assess the abundance of the acidobacterial and total bacterial communities in each of 40 bulk soil samples and 20 mesocosm samples from soybean rhizosphere soil. As standard, amplicons of *Acidobacteria capsulatum* (DSMZ 11244) and bacterial clone from an environmental sample were obtained by PCR using primers pA-F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards *et al.*, 1989) and 1378R (5′-CGGTGTGTACAAGGCCCG GGAAGG-3′) (Heuer *et al.*, 1997), purified (QIA-quick PCR purification kit; Qiagen, Venlo, the Netherlands), and ligated into the pGEM-T vector (Promega, Leiden, the Netherlands). Ligation products were transformed with *Escherichia coli* JM109-competent cells (Promega). Cloned inserts were re-amplified using primers SP6 and T7, and plasmid DNA was isolated (QIAprep Spin Miniprep Kit; Qiagen) from appropriate clones (i.e. belonging to the desired target group). DNA standard curves were generated by dilution series of 10^3 to 10^8 copies μL^{-1} using duplicate 10-fold dilutions of isolated plasmid DNA. For qPCR of 16S rRNA gene fragments from *Acidobacteria* and total bacteria, Acid31 (5′-GATCCTGG CTCAGAATC-3′) (Barns *et al.*, 1999)/Eub518 (5′-ATTA CCGCGGCTGCTGG-3′) (Muyzer *et al.*, 1993) and Eub338 (5′-ACTCCTACGGGAGGCAGCAG-3′) (Lane, 1991)/Eub518 primer pairs were used, respectively. Each 25-μL reaction mixture contained 12.5 μL absolute qPCR SYBR green 2 × reaction mix (Abgene, Epsom, UK), 1.25 μL of each primer (30 μM), 2.5 μL bovine serum albumin (10 mg mL^{-1}), and 50 ng template DNA. All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). PCR conditions for *Acidobacteria* and total bacteria were carried out as described by Fierer *et al.* (2005) with the modification of annealing temperature to 49 °C for *Acidobacteria*. PCR amplifications and product quantification were performed using the Rotor-Gene 3000 (Corbett Research). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Automated analyses of PCR amplicon quality (e.g. PCR baseline subtraction, Ct threshold setting to the linear amplification phase) and quantity were performed using ROTOR-GENE 6 software (Corbett Research). Statistical analyses of qPCR data were performed using the STATISTICA 9 package (StatSoft Inc., Tulsa, OK). One-way ANOVA was used to determine the significance of the differences between all soil samples. The comparison of soil samples was based on *post hoc* analysis using Tukey's HSD test. Correlations were calculated to test the relationships between acidobacterial relative abundance and soil factors by using the 'multtest' package in R (version 2.6.0; The R Foundation for Statistical Computing).

Amplification and sequencing of 16S rRNA gene fragments

Primers targeting the V4 region of bacterial 16S rRNA gene were used for the amplification. Adapter sequence was added to the primers as recommended by Roche; barcodes of 8 bp and AC linker (added to forward primers only); and the primer sequence 5′-AYTGGGYDTA AAGNG-3′ for the forward primer and 5′-CCGTCAATT CMTTTRAGT-3′ for the reverse primer (http://pyro.cme.msu.edu/). DNA template in PCR consisted of pools by mixing aliquots from 15 DNA subsamples from bulk soil (three replicates of soil DNA extraction for each of five soil samples) for each sampling site and period or 15 DNA subsamples (three replicates of soil DNA extraction for each of five soybean rhizosphere soil samples) for each replicate of mesocosm experiment. PCR was performed in duplicate for each pooled DNA sample (samples are described in Fig. 1). The PCR mixture contained 2.5× reaction buffer, 0.2 mM of each dNTP, 1 μM of each primer (Alpha DNA, Montreal, Canada), 10 ng of

template DNA, and 0.056 U of FastStart High-Fidelity PCR System enzyme blend (Roche Applied Sciences, Indianapolis, IN). The following conditions were chosen for amplification after optimization: initial denaturation for 5 min at 95 °C; 27 cycles of 45 s at 95 °C, 45 s at 57 °C, and 1.5 min at 72 °C; and final extension for 10 min at 72 °C. After gel analysis, amplicons from 10 reactions were combined and purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA). PicoGreen base quantification allowed the normalization for the construction of multiplex amplicon pools (Harris *et al.*, 2010). The samples were sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT).

Sequence analysis

Sequences and quality information were extracted from the Standard Flowgram Format (SFF) files using the SFF converter tool in the Galaxy interface (Goecks *et al.*, 2010). Sequences were analyzed using the Qiime version 1.2.1 scripts (Caporaso *et al.*, 2010), which were made available in the Galaxy interface. Firstly, all the sequences that had a perfect match to the primer sequences contained no homopolymer run exceeding six nucleotides and without ambiguous characters were assigned to samples by matching to barcode sequences. Reverse complementary sequences were put in the forward orientation and assigned to samples by partially matching to barcode sequences. This step was confirmed by V-REVCOMP tool using hidden Markov models (Hartmann *et al.*, 2011). Secondly, sequence errors introduced during pyrosequencing were detected using the option for titanium data in the DENOISER 0.91 program (Reeder & Knight, 2010). The sequences were also checked for PCR chimeras using UCHIME version 4.2.40 (Edgar *et al.*, 2011). Only sequences with a length between 200 and 350 base pairs were accepted, and bases with a quality score lower than 20 were trimmed. The low-quality sequences were discarded. The obtained high-quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST version 1.2.21 (Edgar, 2010) with a minimum sequence identity cutoff of 97%. For each OTU, the most abundant sequence was selected as a representative for all sequences within an OTU. Taxonomy was assigned to the representative sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4), with a minimum support threshold of 60%. Finally, the OTU table was filtered for specific taxonomic terms using scripts provided by Qiime. Relative abundances of *Acidobacteria* were estimated in each bacterial community by comparing the number of sequences classified as belonging to the *Acidobacteria* vs. the number of classified bacterial sequences per sample. The relative abundance of *Acidobacteria* subgroups across all individual samples, in turn, was estimated by comparing the number of sequences classified as belonging to each *Acidobacteria* subgroup vs. the number of classified acidobacterial sequences. The classified acidobacterial sequences were deposited in MG-RAST under accession numbers 4497240.3 to 4497251.3 (list of public access codes can be found in Supporting Information, Table S1). Explicit relationship between relative abundance of *Acidobacteria* subgroups and bulk soil factors from cropland and forest sites was examined by constrained ordination generated by redundancy analysis (RDA) performed using CANOCO 4.5 (ter Braak & Šmilauer, 2002). Spearman's rank correlation coefficients were calculated to explore the relationship between relative abundance of *Acidobacteria* subgroups and soil factors referent to different sampling sites by using the 'multtest' package in R (version 2.6.0; The R Foundation for Statistical Computing). *P* values were corrected for multiple testing, using the false discovery rate controlling procedure (Benjamini & Hochberg, 1995).

Results

Bulk soil physicochemical factors

The soils contained 43–50% sand, 4–6% silt, and 46–53% clay in all sites. Based on ANOSIM of the physicochemical factors, bulk soil from cropland and adjacent forests formed distinct groups, which groups were confirmed by significant *R*-value ($R = 0.955$, $P < 0.0001$). Soil pH ranged from 3.7 to 4.7 in forest soils and from 5.0 to 5.7 in the majority of the cropland soils. The detected levels of Al, Al saturation, and Fe were respectively 10, 8, and 3 times higher in forest soils than in cropland soils, except for the bulk soil from cropland BS-C1 (Fig. 1; Table S2). Sum of bases (SB) and base saturation (V) were higher in cropland soils than in forest soils (Table S2). The results showed that soil pH was negatively correlated ($P < 0.05$) with Al saturation, potential acidity, Al, Fe, and Mn (Table 1). Both SB and V were negatively correlated with Al ($P < 0.05$), potential acidity ($P < 0.05$), and Al saturation ($P < 0.0005$) (Table 1).

Acidobacterial community abundance

ANOVA was carried out on qPCR data targeting 16S rRNA gene fragment abundances for total bacteria as well as for *Acidobacteria*. These analyses showed significant differences between bulk soils from cropland and adjacent forest ($P < 0.005$) for both the relative and absolute acidobacterial abundance (Table 2). *Acidobacteria* accounted,

Table 1. Spearman's rank correlation matrix for soil chemical attributes of the 0- to 20-cm topsoil layer at different sampling sites

Soil attributes	pH	OM	Al	H + Al	m	P	K	Ca	Mg	SB	CEC	V	B	Fe	Mn	Zn	Cu
pH	–																
OM		–															
Al	−0.850*		–														
H + Al	−0.778*	0.785*	0.857*	–													
m	−0.634*		0.785*	0.785*	–												
P						–											
K			–			0.754*	–										
Ca			−0.785*	−0.785*	−1***	0.850*		–									
Mg			−0.778*	−0.742*	−0.958***	0.801*		0.958***	–								
SB			−0.785*	−0.785*	−1***	0.850*		1***	0.958***	–							
CEC											–						
V			−0.761*	−0.833*	−0.976***	0.826*		0.976***	0.934***	0.976***		–					
B	−0.876*		0.932**	0.748*									–				
Fe	−0.814*		0.904**										0.969**	–			
Mn	−0.757*		0.855*										0.938**	0.988***	–		
Zn																–	
Cu																	–

Significant levels for the Spearman's rank coefficients are indicated at the $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ level.

on average, for 23% in forest soils, 18% in cropland soils, and 14% in soybean rhizosphere of the total bacterial signals. Although the highest *Acidobacteria* 16S rRNA gene content was observed in the bulk soils, the overall abundance of *Acidobacteria* gene copies was not statistically different between bulk soils from cropland and mesocosm samples from soybean rhizosphere (Table 2). The abundance of *Acidobacteria* relative to total bacteria 16S rRNA gene copy numbers was significantly correlated with soil organic matter ($R = 0.627$, $P = 0.00001$), Al ($R = 0.413$, $P = 0.0004$), and pH ($R = -0.407$, $P = 0.008$) (Fig. 2). *Acidobacteria* represented the largest portion of the bacterial community in soils with high organic matter content, Al content, and acidity. Soil Ca levels ($R = -0.482$, $P = 0.001$) and base saturation ($R = -0.494$, $P = 0.001$) were negatively and significantly correlated with the relative abundance of *Acidobacteria*.

Phylum and subgroup-level relative abundance of *Acidobacteria*

Sequence data were examined in soils to estimate the phylum and subgroup relative abundance of *Acidobacteria*. Across the 12 soil samples (samples described in Fig. 1), *Acidobacteria* represented 19% of all classified bacterial sequences detected (32 637 sequences of 175 434 sequences). The relative abundance of acidobacterial sequences within an individual soil bacterial community represented on average 28% in forest soils, 16% in cropland soil, and 17% in soybean rhizosphere.

We further classified *Acidobacteria* DNA sequences into acidobacterial subgroups using the RDP 2 classifier (http://rdp.cme.msu.edu/). The average relative abundances of *Acidobacteria* subgroups for bulk soil from forest and cropland, and soybean rhizosphere soil samples are listed in Table 3, as well as the numbers of OTUs assembled using a 97% similarity for each *Acidobacteria* subgroup. *Acidobacteria* subgroups 1 and 3 were the most abundant in all soils analyzed. RDA of the relative abundance of the *Acidobacteria* subgroups (1–7, 10, and 13) showed that some subgroups (4–7, 10, and 13) were more closely related to cropland soils (Fig. 3). *Acidobacteria* subgroups 1 and 2 were more closely related to forest soil characteristics (Fig. 3). Statistically significant differences were found for the abundances of the acidobacterial subgroups 4 ($P < 0.05$), 6 ($P < 0.05$), and 7 ($P < 0.005$) relative to all *Acidobacteria* in cropland soils vs. the adjacent forest soils (Table 3). Interestingly, the relative abundances of these acidobacterial subgroups (4, 6, and 7) were statistically correlated with different soil factors, specially those linked to soil acidity such as Al and Al saturation, SB, and base saturation (Table 4; Fig. 4). Significant correlations were also found between these subgroups

Table 2. Absolute and relative abundance of *Acidobacteria* in cropland and adjacent forest bulk soils and soybean rhizosphere niche

	Bulk soil – Forest (BS-F)				Bulk soil – Cropland (BS-C)				Soybean rhizosphere (SR)				Statistics	
	BS-F1	BS-F2	BS-F3	BS-F4	BS-C1	BS-C2	BS-C3	BS-C4	SR-I	SR-II	SR-III	SR-IV	BS-F vs. BS-C	BS-C vs. SR
Absolute abundance (qPCR analysis) (10^7 16S rRNA gene copies g soil)														
Total bacteria	3.48a[†] (0.17)[‡]	2.90a (0.08)	6.0A (0.23)	4.59A (0.14)	1.64a (0.01)	2.96a (0.13)	7.12A (0.29)	4.55A (0.15)	2.28a (0.09)	2.89a (0.10)	7.27A (0.22)	6.08A (0.12)	ns[§] (1–2) ns (3–4)	ns (1–2) ns (3–4)
Acidobacteria	0.96a (0.07)[‡]	0.84a (0.02)	1.07A (0.06)	0.85A (0.01)	0.49a (0.05)	0.56a (0.02)	0.74A (0.01)	0.50A (0.005)	0.44a (0.01)	0.45a (0.007)	0.78A (0.008)	0.72A (0.03)	**(1–2) **(3–4)	ns (1–2) ns (3–4)
Relative abundance (%)														
Acidobacteria (qPCR assays)	27.58a (2.61)[‡]	28.96a (1.83)	17.83A (2.72)	18.51A (1.76)	29.87a (2.8)	18.92b (0.12)	10.39A (2.11)	10.98A (1.18)	19.30a (1.10)	15.57a (1.64)	10.72A (1.13)	11.84A (2.53)	*(1–2) **(3–4)	ns (1–2) ns (3–4)
Acidobacteria (Pyrosequencing analysis)	19.92a (4057)[¶]	22.06a (3614)	28.78A (4967)	22.97A (2771)	25.0a (2704)	15.56b (2435)	9.58A (991)	15.16A (1316)	10.43a (1848)	15.03a (3391)	7.90A (2925)	8.72A (1618)	ns (1–2) **(3–4)	*(1–2) ns (3–4)

[†]Values with the same lower- or upper-case letters were not significantly different ($P < 0.05$) based on a Tukey's HSD test.

[‡]Standard deviation of the average for each of five replicates of soil.

[§]Tukey's HSD test was performed separately for field location and sampling period (samples 1–2 and 3–4).

[¶]Total number of acidobacterial reads obtained by barcoded pyrosequencing. Sequences taxonomy assignments were made using RDP 2 classifier (release 10.4).

Significance levels: ns: $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

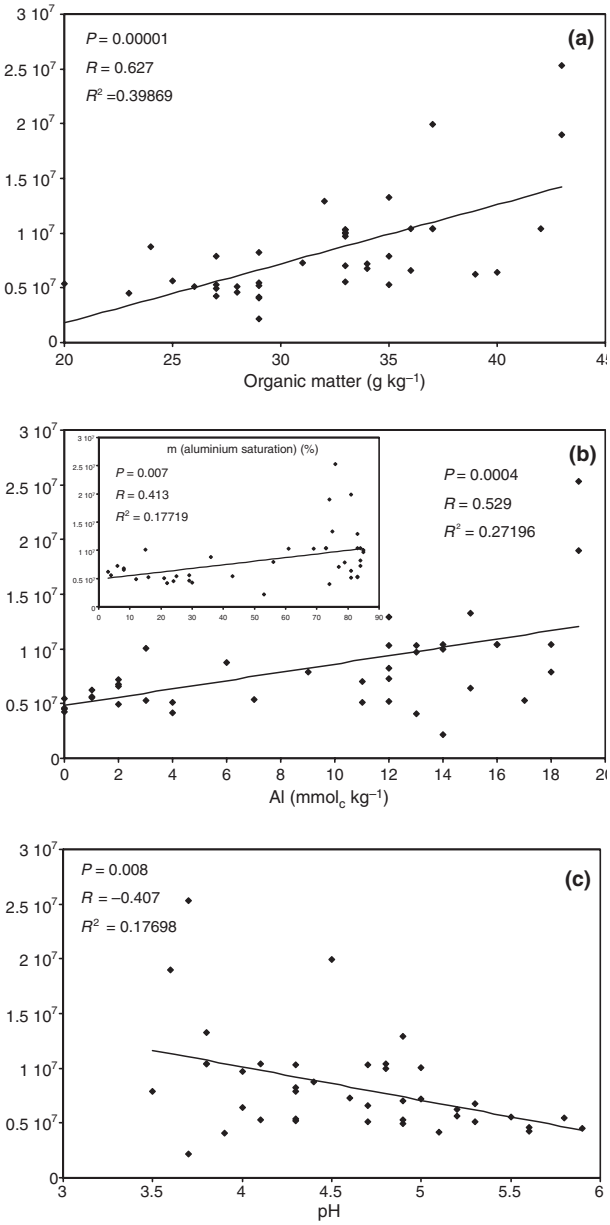

Fig. 2. Effect of (a) organic matter, (b) aluminum (Al) and (c) soil pH on abundance of *Acidobacteria* relative to total bacteria as indicated by the number of 16S rRNA gene copies measured using real-time quantitative PCR assays. Spearman's rank correlations and their statistical significance are depicted. Coefficient of determination (R^2) was calculated.

(4, 6, and 7) and soil Ca and Mg. The dominant *Acidobacteria* subgroup 1 was positively correlated with potential acidity and aluminum saturation, while subgroup 3 was negatively correlated with soil pH (Table 4). *Acidobacteria* subgroups 10 and 13 were detected in all three environments studied. Subgroup 10 was correlated with soil factors linked to soil acidity such as pH, Al, and Al saturation. Moreover, subgroup 13 was correlated with soil P, B, and Zn. Most subgroups appear to be minor constituents of soil acidobacterial communities and often occurring in one or two of the sampled soils; subgroup 11 was detected only in bulk soil from cropland; subgroup 17 was detected in bulk soil from forest and cropland, but not in soybean rhizosphere soil; subgroups 18 and 22 were detected in bulk soil from cropland and soybean rhizosphere soil, but not in forest soils; and subgroup 25 was detected only in soybean rhizosphere soil. No sequences affiliated to *Acidobacteria* subgroups 9, 12, 14–16, 19–21, 23, 24, and 26 were detected in any soil sample (Table 3).

Discussion

The present study assessed the soil acidobacterial community in a Brazilian Amazon region characterized by high rate of deforestation due to the expansion of cropland (mainly soybean) into areas previously covered by native forest. Our results based on qPCR assays and pyrosequencing analyses show that the overall abundance of *Acidobacteria* (relative to all bacteria) differed between soybean cropland and adjacent native forest soils, and they revealed that the differences in soil factors were the major predictors of the variability in relative abundance of *Acidobacteria* subgroups found in these Amazon soils.

Although the overall abundance of *Acidobacteria* significantly differed ($P < 0.005$) between soybean cropland and adjacent forest soils, the pyrosequencing data revealed that not all *Acidobacteria* subgroups responded to this change in land use. Recent studies have demonstrated that changes in soil microbial communities across space are often correlated with the differences in soil chemistry (Frey *et al.*, 2004; Nilsson *et al.*, 2007; Lauber *et al.*, 2008; Jenkins *et al.*, 2009; Kuramae *et al.*, 2011). In particular, it has been shown that the composition, and in some cases diversity, of soil bacterial community is correlated with soil pH (Sait *et al.*, 2006; Eichorst *et al.*, 2007; Hartman *et al.*, 2008; Jenkins *et al.*, 2009; Lauber *et al.*, 2009) and other soil factors than pH, such as Ca/Mg ratio, and Al and phosphorous content (Faoro *et al.*, 2010). This pattern holds both for overall bacterial community composition (Fierer & Jackson, 2006; Baker *et al.*, 2009; Lauber *et al.*, 2009; Kuramae *et al.*, 2010) and for the composition of individual bacterial groups (Nicol *et al.*, 2008; Davis *et al.*, 2009; Jenkins *et al.*, 2009; Jones *et al.*, 2009).

Although previous studies have indicated the effect of soil pH on the abundance of *Acidobacteria* in different soil types (Jones *et al.*, 2009; Rousk *et al.*, 2010) and spatial scales (Lauber *et al.*, 2009), the present study shows differential responses of the *Acidobacteria* subgroups to

Table 3. Abundance of *Acidobacteria* subgroups relative to all *Acidobacteria* and numbers of OTUs of acidobacterial subgroups in cropland and adjacent forest bulk soils and soybean rhizosphere niche

	% of total acidobacterial sequences (range)			OTUs (range)			Statistics (% of total acidobacterial sequences)		Statistics (OTUs)	
Subgroups	BS-F	BS-C	SR	BS-F	BS-C	SR	BS-F vs. BS-C	BS-C vs. SR	BS-F vs. BS-C	BS-C vs. SR
1	51.4 (40.1–65.2)[†]	32.8 (30.9–36.5)	41.0 (29.5–60.7)	42 (41–43)	41 (34–48)	45 (41–49)	ns[‡]	ns	ns	ns
2	11.2 (8.0–13.9)	7.2 (6.7–7.6)	5.3 (2.9–7.8)	16 (12–23)	16 (10–23)	12 (8–15)	ns	ns	ns	ns
3	29.0 (20.1–51.2)	29.6 (17.7–51.2)	30.8 (22.8–40.3)	27 (19–34)	32 (25–41)	39 (23–54)	ns	ns	ns	ns
4	0.1 (0.0–0.4)	4.6 (0.58–7.3)	5.4 (0.7–10.5)	2 (0–4)	18 (7–27)	11 (3–18)	*(1–2)/*(3–4)	ns	*(1–2)/*(3–4)	ns
5	4.0 (3.0–5.3)	3.8 (1.0–6.0)	1.2 (0.4–1.6)	5 (4–7)	6 (4–8)	5 (4–6)	ns	ns	ns	ns
6	2.5 (1.6–3.1)	16.6 (5.4–23.1)	13.1 (5.6–20.3)	6 (5–7)	10 (6–13)	13 (7–19)	*(1–2)/*(3–4)	ns	*(1–2)/*(3–4)	ns
7	< 0.1 (0.0–0.04)	1.8 (0.5–3.3)	1.4 (0.1–2.8)	1 (0–1)	6 (4–7)	4 (1–6)	**(1–2)/*(3–4)	ns	**(1–2)/*(3–4)	ns
8	< 0.1 (0.0–0.02)	< 0.1 (0.0–0.07)	< 0.1 (0.0–0.05)	1 (0–1)	1 (0–1)	1 (0–2)	ns		ns	
9	ND	ND	ND	ND	ND	ND				
10	0.1 (0.04–0.2)	0.3 (0.2–0.4)	0.3 (0.0–0.9)	3 (1–5)	3 (1–5)	3 (0–7)	ns	ns	ns	ns
11	ND	< 0.1 (0.0–0.1)	ND	ND	1 (0–2)	ND				
12	ND	ND	ND	ND	ND	ND				
13	1.3 (0.9–1.7)	2.3 (1.5–3.3)	1.3 (0.8–1.9)	7 (5–9)	6 (5–8)	4 (3–7)	ns	ns	ns	ns
14	ND	ND	ND	ND	ND	ND				
15	ND	ND	ND	ND	ND	ND				
16	ND	ND	ND	ND	ND	ND				
17	< 0.1 (0.0–0.2)	0.5 (0.0–1.3)	ND	1 (0–2)	4 (0–7)	ND	ns		ns	
18	ND	< 0.1 (0.0–0.1)	< 0.1 (0.0–0.2)	ND	1 (0–2)	1 (0–3)		ns		ns
19	ND	ND	ND	ND	ND	ND	ns		ns	
20	ND	ND	ND	ND	ND	ND	ns		ns	
21	ND	ND	ND	ND	ND	ND	ns		ns	
22	ND	0.1 (0.09–0.5)	< 0.1 (0.0–0.02)	ND	1 (0–4)	1 (0–1)		ns		ns
23	ND	ND	ND	ND	ND	ND				
24	ND	ND	ND	ND	ND	ND				
25	ND	ND	< 0.1 (0.0–0.05)	ND	ND	1 (0–1)				
26	ND	ND	ND	ND	ND	ND				

BS-F, bulk soil from forest; BS-C, bulk soil from cropland; SR, soybean rhizosphere.

[†]Range (%) of the average for each of four replicates of soil.

[‡]Tukey's HSD test was performed separately for field location and sampling period (samples 1–2 and 3–4).

SR. Significance levels: ns: $P > 0.05$; *$P < 0.05$; **$P < 0.005$.

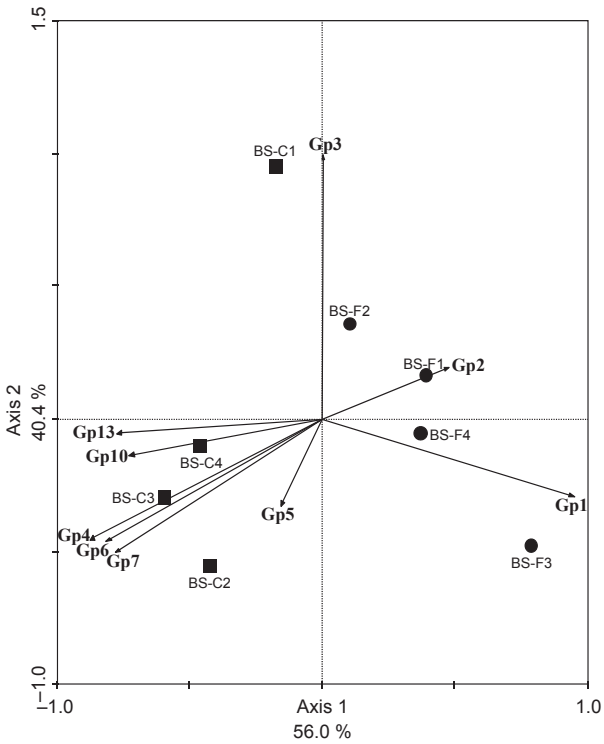

Fig. 3. Constrained ordination diagram for sample plots (= bulk soil from forest; = bulk soil from cropland) in the first two RDA axes based on the soil chemical characteristics of the different sampling sites and their relationship with the relative abundance of *Acidobacteria* subgroups (1–7, 10, and 13). Each vector points to the direction of increase for a given acidobacterial subgroup (Gp), and its length indicates the strength of the correlation between this variable and the ordination scores. Bulk soil (BS) samples were taken before sowing soybean seeds (BS-C1 and BS-C3) and after soybean harvest (BS-C2 and BS-C4) in cropland sites. Concomitantly, bulk soil samples were taken in adjacent forests (BS-F1 and BS-F3) and (BS-F2 and BS-F4).

other soil characteristics (including soil factors not measured in previous studies, such as Al, Ca, Mg, K, B, and micronutrients) than soil pH altered probably by liming practice required in the agriculture management into Amazon forest soils. In the soil environment, pH is a master variable and it is related to changes in other soil factors, such as Al concentration and nutrient availability (McBride, 1994). Aluminum toxicity has long been known to affect microorganisms as well as plants in tropical soils (Wood, 1995; Joner *et al.*, 2005). The soil in the region where our samples were collected is rich in Al, in particular the forest soils. Hence, it is not surprising that Al and pH would covary and correlate with acidobacterial community in these native soils. In addition, bacterial communities dominated by *Acidobacteria* phylum changed significantly along gradients of base saturation, Al, and pH in Western Amazon soils (Jesus *et al.*, 2009).

Liming practice in agriculture is based on the calculation of the necessity of Ca and Mg by the plant, tolerance to aluminum and clay content. In acid tropical soils, changes in soil chemical properties due to lime requirement for agricultural practices include a decrease in H^+ activity, a decrease in Al, Al saturation, and Mn toxicities, an increase in Ca and Mg availability, and benefits associated with Ca as a complementary ion on the cation exchange complex (Abruña *et al.*, 1964; Amedee & Peech, 1976; Oliveira & Pavan, 1996). This practice appears to have direct effect on *Acidobacteria* subgroups 4, 6, and 7 because the abundances of these subgroups were negatively correlated with Al and Al saturation. However for subgroup 6, Ca and saturation of bases were additional factors, while for subgroup 7, Mg and SB (K, Ca, Mg) were additional factors explaining their high abundance in cropland soils. Such elements (Mg, K, Ca) are required for the growth of all living organisms. Magnesium ions are required by large number of enzymes for their catalytic action, including all enzymes utilizing or synthesizing ATP, or those that use other nucleotides to synthesize DNA and RNA. However, the ionic magnesium cannot directly be uptaken by the biological membranes because they are impermeable to magnesium (and other ions), so transport proteins must facilitate the flow of magnesium and other ions, both into and out of cells (Bevenbach, 1990). Future studies on *Acidobacteria* subgroups 4, 6, and 7 functions certainly will elucidate the role of those subgroups in soil.

Differential responses of *Acidobacteria* subgroups to abiotic soil factors are probably due to the lifestyles of these microorganisms in soils (Ward *et al.*, 2009). When these responses are related to specific factors that are involved in soil management, they may be used to develop early-warning bioindicators for soil effects, for instance, due to agricultural management of Amazon forest soils into soybean cultivation. According to McCarty & Munkittrick (1996), bioindicator is an anthropogenically induced response in biomolecular, biochemical, or physiological parameters that has been causally linked to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization. Our findings about differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes. However, there are still not many studies focusing on responses of acidobacterial subgroups to environmental factors. Ultimately, a better understanding of how agricultural management affects soil microbial ecology will support the development of more productive, sustainable systems.

In this study, biological effects at the taxonomical acidobacterial subgroup level were detected using primer set

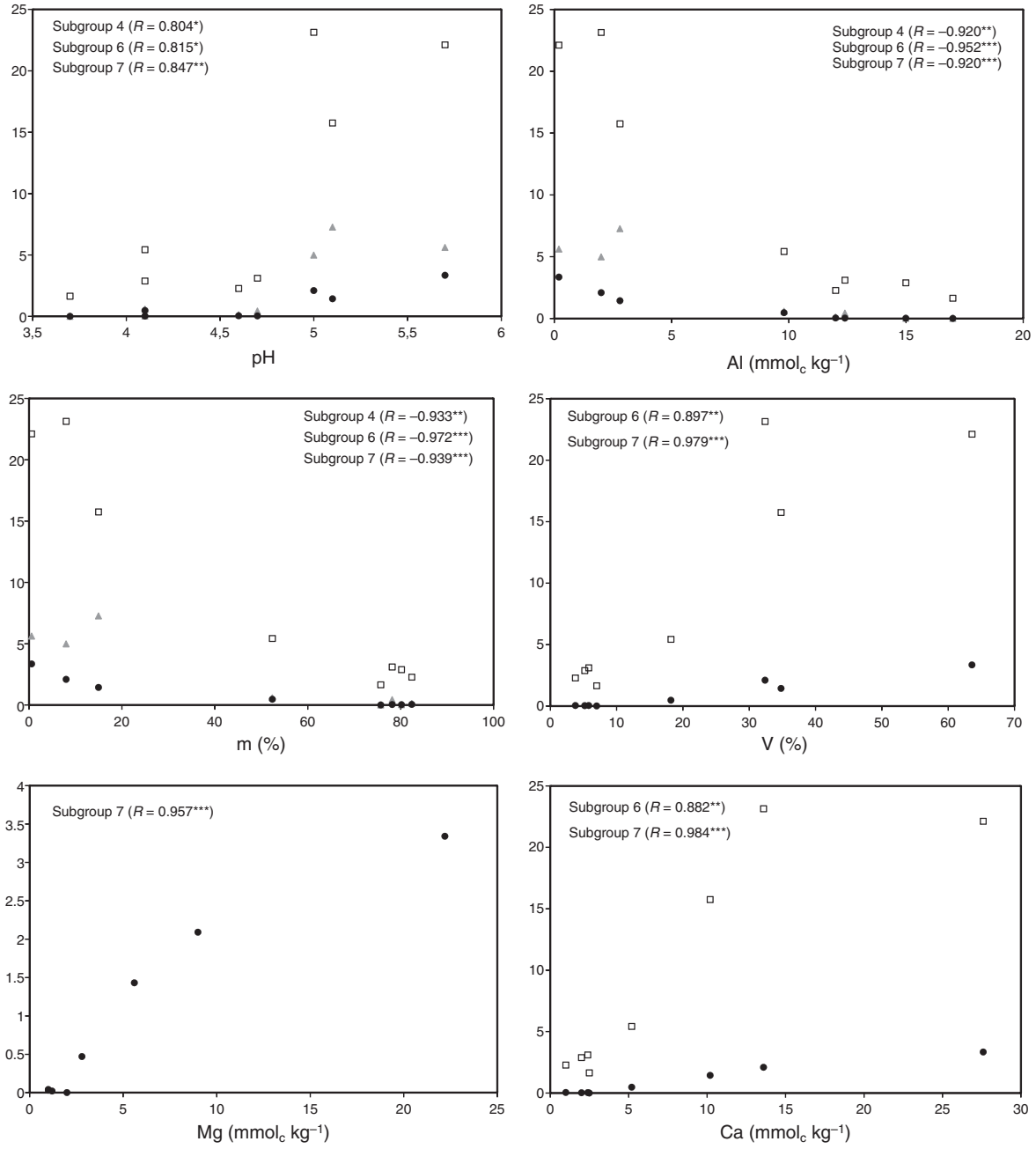

Fig. 4. Effect of soil pH, Al, Al saturation (m), base saturation (V), Ca, and Mg on abundance of *Acidobacteria* subgroups (4, 6, and 7) relative to all *Acidobacteria* using pyrosequencing data. Triangles (gray) represent *Acidobacteria* subgroup 4; squares (no filled) represent *Acidobacteria* subgroup 6; circles (black) represent *Acidobacteria* subgroup 7. Spearman's rank correlations between subgroup abundance and each soil factor are depicted. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

'universal' targeting the V4 region of bacterial 16S rRNA gene. In our soil samples were detected 15 different acidobacterial subgroups among the 26 acidobacterial subgroups classified – subgroups 1–8 according to Hugenholtz *et al.* (1998); subgroups 9–11 according to Zimmermann *et al.* (2005); and subgroups 12–26 according to Barns *et al.* (2007). Many researchers have pointed out that not all acidobacterial subgroups could be detected using group-specific primers, such as Acid31F (Barns *et al.*, 1999) and ACIDO (Lee & Cho, 2011) designed for the selective amplification and detection of members of the phylum *Acidobacteria*. According to Barns *et al.* (2007), George *et al.* (2009), Jones *et al.* (2009), Kielak *et al.* (2009), Sait *et al.* (2006), Lee & Cho (2011), the

Table 4. Spearman's rank correlation coefficients and statistical significance between abundance of *Acidobacteria* subgroups (1–7, 10, and 13) relative to all *Acidobacteria* and soil factors

	Acidobacteria subgroups								
Soil factors	1	2	3	4	5	6	7	10	13
pH	−0.304	−0.437	−0.707*	0.804*	0.375	0.815*	0.847**	0.710*	0.484
OM	0.347	0.558	−0.130	−0.427	0.221	−0.290	−0.375	−0.706	−0.143
Al	0.619	0.691	0.446	**−0.920****	−0.223	**−0.952*****	**−0.920*****	−0.807*	−0.669
H + Al	0.737*	0.610	0.117	−0.810*	−0.134	−0.769*	−0.826*	−0.841*	−0.465
m	0.737*	0.675	0.344	**−0.933****	−0.259	**−0.972*****	**−0.939*****	−0.723*	−0.666
P	−0.587	−0.553	−0.114	0.541	−0.069	0.705	0.478	0.352	0.802*
K	−0.410	−0.482	0.011	0.274	−0.198	0.489	0.305	−0.066	0.477
Ca	−0.530	−0.503	−0.459	0.767*	0.265	**0.882****	**0.984*****	0.543	0.330
Mg	−0.433	−0.455	−0.485	0.685	0.247	0.822*	**0.957*****	0.451	0.208
SB	0.496	−0.493	−0.469	0.736*	0.251	0.866*	**0.978*****	0.499	0.288
CEC	0.342	0.187	−0.547	−0.124	0.179	0.142	0.226	−0.540	−0.281
V	−0.608	−0.575	−0.399	0.845*	0.280	**0.897****	**0.979*****	0.653	0.402
B	0.448	0.642	0.395	−0.690	0.032	−797*	−0.666	−0.683	−0.750*
Fe	0.256	0.736*	0.327	−0.597	0.194	−0.606	−0.538	−0.686	−0.516
Mn	0.293	0.748*	0.589	−0.796*	−0.155	−0.792*	−0.673	−0.660	−0.653
Zn	−0.670	−0.326	−0.008	0.455	0.046	0.644	0.427	0.391	0.810*
Cu	−0.160	0.614	0.234	−0.242	0.356	−0.664	−0.158	−0.033	−0.038

Bold values indicate a significant difference using Bonferroni correction.
Coefficient of determination (R^2) for significant correlations can be found in Table S3.
Significant levels for the Spearman's rank coefficients are indicated at the *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ level.

two group-specific primers (Acid31F and ACIDO) do not detect the subgroups 2, 22, and 25, which subgroups were accounted in this study by using the 'universal' V4 region primers. In our soil samples, acidobacterial subgroup 2 was one of the most dominant. The acidobacterial subgroups 1–8 identified in this study were also ubiquitous and abundant members of soil acidobacterial communities in soil surveys worldwide (Barns *et al.*, 1999; Janssen 2006; Jones *et al.*, 2009; Rousk *et al.*, 2010). In our analysis, acidobacterial subgroups 4, 6, and 7 showed preponderant responses to abiotic soil factors which were clearly affected by the changes in soil use from native forests into soybean production fields, such as Al, Ca, Mg, Mn, and B.

In conclusion, the acidobacterial community responds to agricultural management of Amazon forest soils into soybean production sites, through the effects on various soil factors, not only those related to soil acidity. The differential responses of the *Acidobacteria* subgroups to specific abiotic soil factors can help to reveal what drives their population changes in Amazon soils recently converted into cultivation and open the possibilities to explore acidobacterial subgroups as bioindicators for agricultural soil management effects in the wide Amazon area.

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Authors' contribution

A.A.N. and E.E.K. share first authorship.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of public access codes of the clean acidobacterial sequences uploaded to the metagenomic RAST (MG-RAST) server.

Table S2. Soil chemical factors of the 0 to 20 cm topsoil layer at different sampling sites.

Table S3. Coefficient of determination (R^2) values for significant correlations between relative abundance of acidobacterial subgroups and soil factors.

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