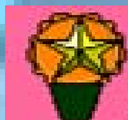
African Journal of Microbiology Research

Volume 9 Number 40, 7 October, 2015
ISSN 1996-0808



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Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7:3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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academicJournals

Vol. 9(40), pp. 2103-2110, 7 October, 2015
DOI: 10.5897/AJMR2015.7440
Article Number: E7B851E55924
ISSN 1996-0808
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African Journal of Microbiology Research

Review

Prevalence of *Mycobacterium leprae* in the environment: A review

Elderson Mariano de Souza VALOIS[1]*, Franciely Maria Carrijo CAMPOS[2] and Eliane IGNOTTI[2]

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Received 20 February, 2015; Accepted 1 September, 2015

The purpose of this review was to study the presence of *Mycobacterium leprae* in the environment and its relation with meteorological variables such as temperature and humidity. There are reports, which provide evidence that meteorological factors such as temperature and soil humidity can influence the dynamics of *M. leprae*. However, leprosy cases are registered both in the rainy and dry seasons, indicating that *M. leprae* remains viable in different environmental conditions. Therefore, it is difficult to establish the meteorological pattern(s) required to maintain the bacilli in the environment. The extensive area of endemic countries, endemicity in the bordering countries, diversity of biomes, and lack of urban infrastructure together with weather features are possible factors that influence transmission of the disease.

Key words: Leprosy, environmental health, molecular biology.

INTRODUCTION

Leprosy is a chronic infectious disease caused by the bacillus *Mycobacterium* (*M.*) *leprae*. The disease, which is prevalent in most tropical and subtropical regions of the world (World Health Organization (WHO), 2014), can manifest itself in different clinical forms depending on the type of host immune response.

In 2011, the WHO published the Enhanced Global Strategy for minimizing the leprosy burden, in order to reduce the disease incidence and its physical, social, and economic consequences. Brazil and India are responsible for 90% of the leprosy cases in the world. In 2012, 232,857 new cases of leprosy were registered worldwide. Regions with the highest number of detected cases are Southeast Asia (71%), the Americas (15.5%), India (134,752 cases), and Brazil (33,303 cases) according to the WHO (2013).

The transmission mechanism for leprosy remains unclear, despite it being studied for centuries. For a long time, it was believed that the only source of transmission of *M. leprae*, the main etiologic agent, was multibacillary patients not receiving treatment. There are, however, a considerable number of epidemiological and microbiological observations indicating that environmental sources (Loughry et al., 2009) can also play an important role in transmission of the disease by indirect contact (Kadza, 2000).

Molecular biological studies have revealed the presence of bacilli in the environment. These findings

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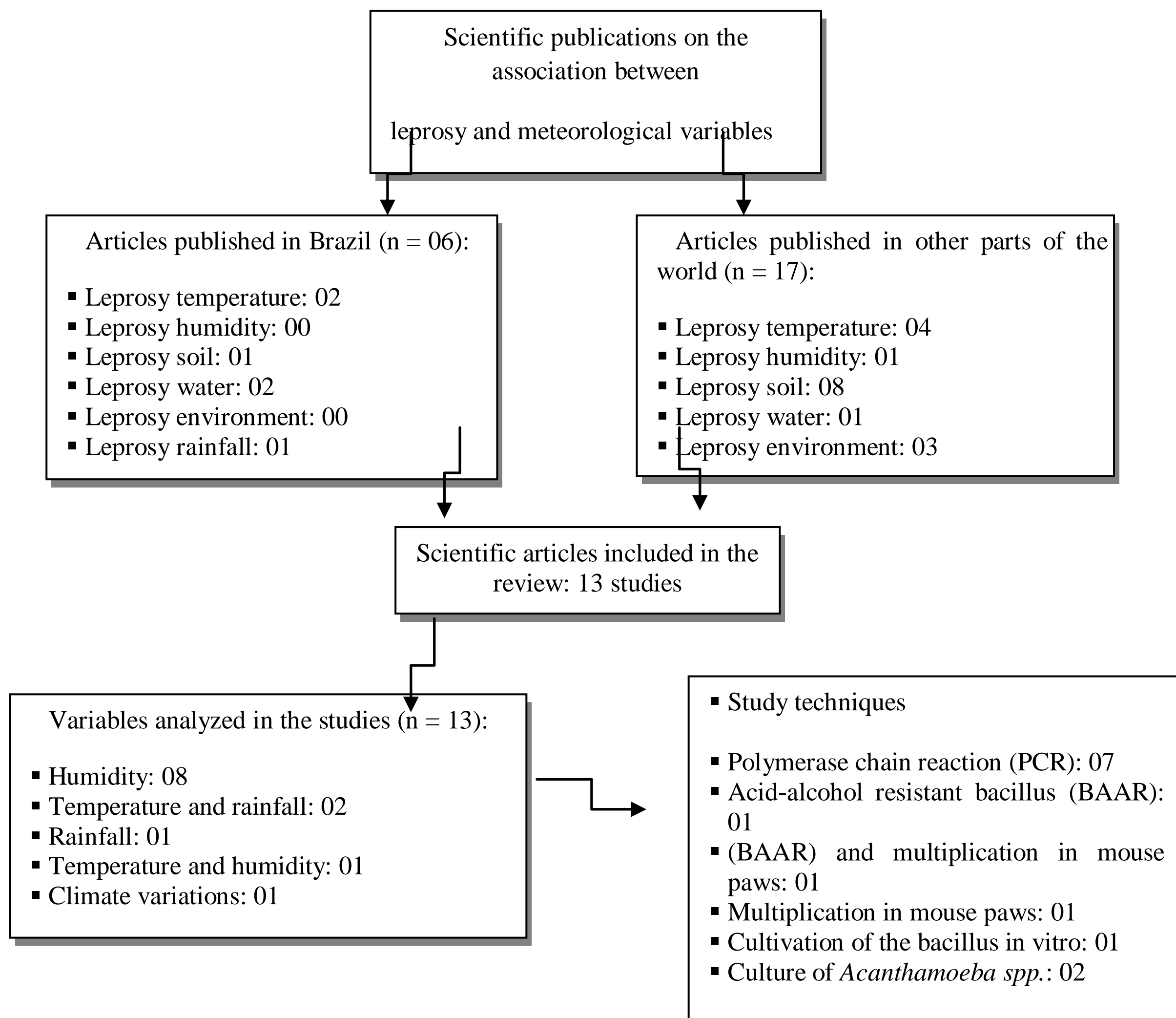

Figure 1. Flowchart of the literature review process: publications from 1980–2014 on the environmental prevalence of *M. leprae* and its association with meteorological variables.

strengthen the hypothesis of transmission of the disease independent of contact with patients, and/or maintenance of viable bacilli in the environment for long periods. As a corollary, meteorological conditions in the environment that favor the maintenance and viability of the bacilli must also be important to the disease transmission. To evaluate this hypothesis, we analyzed existing scientific literature on the presence of *M. leprae* in the environment, and its relation with meteorological variables.

LEPROSY RESEARCH: FUTURE TARGETS AND PRIORITIES

Of the 13 original articles on the association between *M. leprae* and the environment, eight involved relative humidity (%), and one each involved: temperature (°C) and rainfall (mm), only rainfall, temperature and humidity, and culture of *Acanthamoeba castellanii* and climate variations (Figure 1).

In the 1980s, studies aimed to find possible relationships between the environment and *M. leprae*. One of the techniques used in this period was Ziehl-Neelsen (ZN) staining, which is specific for acid-alcohol-resistant bacilli (BAAR) and non-cultivable acid-fast bacilli (NCAFB) (Salem and Fonseca, 1982; Kadza, 1981).

ZN staining is a bacilloscopic procedure that effectively stains acid-alcohol resistant mycobacteria; the staining intensity varies with the species of mycobacterium the microorganisms obtained from the soil or water samples (Wahyuni et al., 2010).

From 1980 to 1990, viability of the bacilli was tested under different environmental conditions. The specificity of the bacilli was determined using a multiplication method of *M. leprae* in mouse paws. Shepard in 1960

revealed their viability, the monitoring tests chemotherapeutic and levels of drug resistance using inoculation of *M. leprae* in the footpads of normal and immune-compromised mice (Azulay et al., 2008).

In 2000, research focusing on cultivation of the bacillus *in vitro* was unsuccessful, although some studies have shown evidence of metabolic activity *in vitro* (Levy and Ji, 2006).

Genome analyses of the mycobacterium have shown that cultivation on artificial media is not possible. This is because even less than half of the genome contains functional genes; the majority consists of inactivated or pseudo genes. Moreover, the genome has undergone progressive reduction, accompanied by genetic degradation and a decrease in size. These evolutionary changes originated with the elimination of important metabolic pathways and related ancillary functions of *M. leprae*, particularly those involved in catabolism (Levy and Ji, 2006).

The absence of experimental models that mimic the disease in humans, and the inability to grow *M. leprae in vitro* represent historically important limitations in the development of appropriate tools for the control of leprosy. However, owing to advances in molecular biology techniques, many studies on the *M. leprae* genome have been conducted (Silvestre, 2011).

From 2000 onwards, amplification of specific DNA sequences of the bacillus became possible by polymerase chain reaction (PCR). This technique was advantageous in that it required small numbers of the bacilli and was highly sensitive (Donoghue et al., 2001).

Recent publications on the genome sequences of *M. leprae, M. tuberculosis*, *M. bovis* and *M. smegmatis*, along with the almost-complete sequences of several other mycobacterial species (*M. avium*, *M. marinum*, *M. paratuberculosis,* and *M. ulcerans)* have enabled the identification of unique and specific proteins in *M. leprae* (Cole et al., 2001; Geluk et al., 2005).

The main method carried out in the study comprised PCR of samples from soil and water, by having high sensitivity of the bacillus, since the sequences of ribosomal RNA (rRNA) (Silvestre, 2011; Donoghue et al., 2001).

New typing methods to conclusively identify *M. leprae* have evolved with the technique of multiple-locus value analysis (MLVA). This technique ensures greater genetic differentiation in a wide range of samples with allelic diversity within a community, and thus, is useful in the detection of leprosy transmission (Young et al., 2004; Groathouse et al., 2004; Zhang et al., 2005).

Table 1 shows studies on *M. leprae* in the environment and its relation with meteorological variables published between 1980 and 2014, presented in chronological order and by the variables analyzed. In terms of temporal evolution, the highest number of studies has been published since 2000, the majority being conducted in India.

Further, analyses of soil samples have shown that *M. leprae* also has non-human reservoirs such as armadillos and protozoans. Moreover, environments favorable to pathogen survival, such as water, soil, sphagnum, as well as other factors are propitious to its transmission (Desikan and Sreevatsa, 1995; Truman, 2005; Turankar et al., 2012). The presence of *M. leprae* in water sources reflects its association with protozoans or invertebrate hosts, as well as some free-living mycobacteria (Whan et al., 2006).

Studies on free-living amoebae have revealed an association with water consumed by the population, and in some cases, with treated water (Falkinham et al., 2001). Wheat et al. (2014) showed that *M. leprae* can remain viable long-term in environmental ubiquitous free-living amoebae and retain the virulence in mouse model.

M. leprae can survive outside its main host in free-living protozoans as *Acanthamoeba castellanii* for 4 days without apparent difficult. These results show that free-living terrestrial or water-borne protozoans can act as "wild macrophages," facilitating survival of the bacilli in the environment when expelled from the human host (Lahiri and Krahenbuhl, 2008). A recent experimental study verified that *M. leprae* remains viable for up to eight months within amoebic cysts (Wheat et al., 2014).

Multibacillary patients spread the leprosy bacilli through their nasal secretions, which in tropical regions remain viable for up to 9 days, and up to 46 days in moist soil at room temperature (Desikan, 1997). In the province of Maluku, Indonesia, where leprosy is endemic, 27% of the villagers were found to carry the bacillus within their nasal cavities (Izumi et al., 1998).

A study carried out in West Bengal, India, in 2009 analyzed 207 soil samples in areas with active cases of leprosy. *M. leprae* was viable in 28 of these samples. Single nucleotide polymorphism (SNP) testing of the bacilli found in both the environment and in patients revealed that they were of the same genotype. The study demonstrated the potential role of viable bacilli in the environment as a source of disease transmission (Turankar et al., 2012). However, it had limitations with regard to identifying the metabolic activity of the bacilli, as well as mechanisms of extended survival and transmission of *M. leprae* in different environments. Furthermore, it was observed that the proportion of samples with evidence of *M. leprae* was higher in humid areas (Izumi et al., 1998; Desikan, 1997). These findings indicate that humidity and rain helps the bacilli to survive for longer periods in the environment.

In a study conducted in Ghatampur, India, in 2008, 80 soil samples were collected, of which 40 were from residential areas housing leprosy patients, while the other 40 were from places with no patients identified (control). Of the 28 soil samples positive for viable *M. leprae*, 22 were from the residential areas, while 6 were from the control areas. Thus, the bacilli released by patients during coughing and sneezing can survive for varying periods depending on the environmental conditions. This

Table 1. Studies on the presence of *M. leprae* in the environment and its relationship with meteorological variables, published between 1980 to 2014.

Reference year of publication	Place and time of study	Variable and technique	Main findings
Humidity			
Wahyuni et al., 2010 Indonesian Journal of Tropical and Infectious Disease	Java, Indonesia 2008	Humidity PCR	Positive results in 22/90 water samples collected, 11 water samples, collected from wells that were never used by leprosy cases, were also positive.
Adriaty et al., 2010 Indonesian Journal of Tropical and Infectious Disease	Island Poteran, Sumenep, Madura and East Java, Indonesia 2009	Humidity PCR	201 samples of *M. leprae*, 91 collected from wells; 26.4% samples PCR-positive. The water used for clinical leprosy groups showed positive PCR in samples, and groups without the disease who used this water were more susceptible to leprosy.
Turankar et al., 2012 Infection, Genetics and Evolution	West Bengal, India 2009	Humidity PCR	Samples, both from the environment (soil) and the multibacilary patients exhibited the same genotype when tested by single nucleotide polymorphism (SNP) typing.
Temperature and Humidity			
Desikan e Sreevatsa, 1995 Leprosy Review	Agra, India 1993	Temperature Humidity Multiplication of *M. leprae* in mouse paws	Between the months of March and April, with temperatures between 24-33°C and atmospheric humidity of 44-28%, the bacilli survived for 14 days. During the monsoon season in August and September, with atmospheric humidity between 72-80% and temperatures of 29-33°C the bacilli survived for 28 days. In September and October, with temperatures of 25-32°C and humidity between 66-44%, the bacilli remained viable in the moist soil for 46 days.
Humidity			
Wahyuni et al., 2010 Indonesian Journal of Tropical and Infectious Disease	Java, Indonesia 2008	Humidity PCR	Positive results in 22/90 water samples collected, 11 water samples, collected from wells that were never used by leprosy cases, were also positive.
Adriaty et al., 2010 Indonesian Journal of Tropical and Infectious Disease	Island Poteran, Sumenep, Madura and East Java, Indonesia 2009	Humidity PCR	201 samples of *M. leprae*, 91 collected from wells; 26.4% samples PCR-positive. The water used for clinical leprosy groups showed positive PCR in samples, and groups without the disease who used this water were more susceptible to leprosy.
Turankar et al., 2012 Infection, Genetics and Evolution	West Bengal, India 2009	Humidity PCR	Samples, both from the environment (soil) and the multibacilary patients exhibited the same genotype when tested by single nucleotide polymorphism (SNP) typing.

study further showed that viable and dead organisms can be distinguished using DNA amplification (Mallika et al., 2008).

In another research conducted in Ghatampur and Jalma, known endemic areas of leprosy in India, 18 soil samples, two from each village from different locations near the residences of patients, were examined. The results revealed the presence of *M. leprae* DNA in 33.3%

Table 1. Contd.

Temperature and Humidity			
Desikan e Sreevatsa, 1995 Leprosy Review	Agra, India 1993	Temperature Humidity Multiplication of *M. leprae* in mouse paws	Between the months of March and April, with temperatures between 24-33°C and atmospheric humidity of 44-28%, the bacilli survived for 14 days. During the monsoon season in August and September, with atmospheric humidity between 72-80% and temperatures of 29-33°C the bacilli survived for 28 days. In September and October, with temperatures of 25-32°C and humidity between 66-44%, the bacilli remained viable in the moist soil for 46 days.
Temperature and Rainfall			
Chilima et al., 2006 Applied and environmental microbiology	Karonga, Malawi, Africa 1998 and 1999	Temperature Rainfall PCR	The rates of recovery were consistently higher for dry season samples than for wet season samples of soil. All isolates cultured from soil appeared to be strains of *M. fortuitum* and not M. leprae with a complex pattern for the environmental mycobacterial flora.
Acanthamoeba castellanii			
Lahiri and Krahenbuhl, 2008 Leprosy Review	Laboratory Research Branch, USA 2007	Climate variations	The *Acanthamoeba castellanii* phagocyte showed no apparent adverse effects. The mycobacterium survived for 4 days, thus pointing to the potential role of the amoebae in the protection of *M. leprae* under adverse environmental conditions such as desiccation, and changes in temperature and pH.
Wheat et al, 2014 Plos Negleted Tropical Diseases	Colorado State University and others, USA – 2013/2014.	Climate variations And Virulence	*M. leprae* can remain viable long-term in environmentally ubiquitous free-living amoebae and retain virulence as assessed in the mouse model.

of the soil samples (Mallika et al., 2006).

Between 1998 and 1999, research was conducted in the northern and southern parts of the district of Karonga, Malawi, Africa. Soil samples from 11 villages housing 19 families with a history of leprosy were examined at the end of the dry and rainy seasons. One hundred and thirteen and 35 samples were collected at the end of the dry (1998) and rainy (1999) seasons, respectively, from 10 families. The results from a subset of 32 samples from the same locale, harvested during the dry and rainy seasons, showed the same trends with higher rates of recovery during the dry season (66%) compared with the rainy season (34%). The authors explain that the northern part of the District of Karonga has higher rainfall than the south. This result might be closely linked to climatic changes in the environment, as the bacilli can be removed from the soil and reducing the density of these bacterial population owing to the presence of the excess rainwater. The challenge in the study was the variety of mycobacteria in the soil, which might indirectly influence human health (Chilima et al., 2006). The incidence of leprosy was three times higher in the northern part of the district, which is warmer and more humid than the southern (Fine et al., 1994).

Epidemiological, microbiological, and clinical studies indicate that 50-70% of the sporadic leprosy cases in well-studied populations is reported in people who have had no known contact with other leprosy patients (Chakrabarty and Dastidar, 2002).

The environment can be an alternative transmission pathway for the spread of the disease. *M. leprae* thrives in soil rich in fossil fuels. In 2001, soil samples containing fossil fuels were collected from different parts of the USA, Russia, and Romania. There was a high degree of correlation between the presence of fossil fuels in the soil and leprosy in the countries surveyed. According to the authors, the disease probably occurred due to soil contamination (Chakrabarty and Dastidar, 2002).

In 1981, Kadza conducted a study across nine countries, where 729 samples were collected as follows: 273 from Norway (32.9% positive), 71 from Ivory Coast (23.9% positive), 36 from Portugal (55.6% positive), 20 from India (30.0% positive), 30 from Peru (40.0% positive), and 67 from Louisiana, USA (25.4% positive), 40 from Sweden, 77 from Scotland, and 115 from Germany, all of which were negative for the presence of the bacillus. *M. leprae* from positive samples was inoculated in the footpads of mice and armadillos. Through technique of isolation NCAFB it was possible to show characteristic growth in the footpads of mice and armadillos. The results suggested since more than 30 years that leprosy is transmitted not only by direct contact, but also indirectly by environmental means. However, the researchers could not culture the bacilli using the Lowenstein-Jensen and Middlebrook methods (Kadza, 1981).

A study conducted at the Institute for Leprosy in Agra,

India, found important differences in viability of the bacilli in adverse conditions during dry and rainy seasons. The first experiment was carried out in dry soil in the months of March and April, at temperatures of 24–33°C and atmospheric humidity of 28%. Under these conditions, the bacilli could not survive for more than 14 days. Upon repeating the experiment during the rainy season (August and September) with an atmospheric humidity ranging between 72–80% and temperatures of 29–33°C, the bacilli survived for at least 28 days. In the months of September and October, at temperatures of 25–32°C and humidity between 66–44%, the bacilli remained viable in moist soil for 46 days. Throughout the year, *M. leprae* remained viable for up to five months in soil that was dry, but under the shade. When exposed to direct sunlight for 3 h/day, the bacilli survived for 7 days. Furthermore, the bacilli remained viable for 2 months when stored between 4 and −20°C but when frozen at −70°C, they remained viable for only half the time. When exposed to antiseptics such as Savlon® and alcohol, the bacilli were rapidly killed, while in saline solution at room temperature, they survived for 60 days. These results indicate different survival rates of the bacilli outside the human body under different environmental conditions in India, where the disease is endemic. The transmission by indirect contact and the occurrence of new cases in the absence of known sources is consistent with viable bacilli outside the body. However, the study presented limitations in the management of refrigeration equipment to preserve the bacilli (Desikan and Sreevatsa, 1995).

WATER

Other studies indicate that *M. leprae* can also survive in water. In a study conducted in Poteran Island, Sumenep, Madura, and East Java, Indonesia, 201 samples were collected and divided into three groups: 91 water samples collected from wells, 42 nasal swabs from household contacts, and 68 histological sections from leprosy patients. Upon analyses of the samples, 26.4% isolates from the water sources, 61.9% from the nasal swabs, and 35.3% from the skin biopsies tested positive. PCR results show that water used by leprosy clinics tested positive, and groups without leprosy that used this water were more susceptible to the disease. Therefore, water is considered a possible reservoir and source of infection for leprosy, because detection of *M. leprae* DNA was significantly higher in individuals using the water than in individuals who did not (Adriaty et al., 2010).

Thus, cases of leprosy in individuals with no history of exposure to other known cases might be explained by exposure to viable *M. leprae* in water (Turankar et al., 2012).

Meanwhile, the research in East Java, Indonesia showed that 22 of the 90 samples of water examined were *M. leprae*-positive. Forty-eight samples were collected from wells used by leprosy patients; 11 of these tested positive for *M. leprae*. Interestingly, water samples collected from wells that were never used by leprosy patients also tested positive; *M. leprae* was found in free-living aquatic amoeba-like protozoa. Therefore, existence of the bacilli in water resources used by inhabitants of endemic areas does not seem to be influenced by the presence of leprosy patients living in the same area (Wahyuni et al., 2010).

Finally, the findings of a study conducted in 2002 in an endemic area of Ceará in northeastern Brazil, in the municipalities of Juazeiro, Morada Nova, Sobral, and the state capital Fortaleza, also suggested that infections arise from contact with contaminated bodies of water. The prevalence of infection among individuals using the water for bathing was higher than that among individuals who did not. Therefore, water might be an important carrier of the disease in this region. Streams and rivers have running water only in the rainy season. Thus, when precipitation stops, stagnant pools of water remain and these might serve as potential reservoirs for the bacilli. One limitation of the survey was the small number of counties investigated (Kerr-Pontes et al., 2006).

Molecular-based studies have revealed the importance of meteorological and climatic factors in the life cycle of *M. leprae*. The bacillus is known to remain viable as a probable source of infection leading to disease, especially under conditions of high humidity and temperature that characterize the tropical regions of the world. However, the bacilli can also survive in environments with broad variations in temperature and humidity. Therefore, basic infrastructures including sewers, water supply, and hygiene are the most important factors in protecting against the disease (Silva et al, 2010).

Besides leprosy patients without treatment, those in subclinical stages or those who exhibit spontaneous remissions may also be sources of bacillary spread, providing a transitional period of pathogen excretion via the nasal and/or oral routes (Cree and Smith, 1998).

Literature provides evidences that support the presence of *M. leprae* in the environment, having been found in different abiotic and biotic substrates. It was found in water (Wahyuni et al., 2010) and soil (Mallika et al., 2008) near leprosy clinics. It was also found in sphagnum (Kadza et al., 1980) and in a number of0species ranging from protozoa (Lahiri and Krahenbuhl, 2008) to more complex organisms such as mammals (Truman and Fine, 2010).

The viable bacilli found in water and soil can be an important disseminator of the disease, indicating extra-human sources of *M. leprae*. Locales with moist soil and associated ambient temperatures guarantee the viability of the pathogen (Ooi and Moschella, 2001).

The finding that *M. leprae* can survive ingestion by amoebae suggests that protozoans can significantly improve the survival of these bacilli in the soil, and therefore be instrumental in the transmission of leprosy

(Lahiri and Krahenbuhl, 2008). This shows the potential role of amoebae in the protection of *M. leprae* under adverse environmental conditions such as temperature and pH changes.

The handling and consumption of armadillo meat is also a possible source of *M. leprae* infection, chiefly in patients with no history of contact with other leprosy patients before their diagnosis (Deps et al., 2003). The mechanism of this transmission, however, has not been elucidated yet.

In 2011, a research conducted in Louisiana and Texas, in the southern region of the United States, revealed cases of leprosy in Native Americans who had never been outside the country. The exact mechanism of transmission remains unclear, but armadillos appear to be the possible reservoir, since the patients and the armadillos were shown to carry the same strain of *M. leprae* (Truman et al., 2011).

Before the *M. leprae* genome was decoded in 2001, availability of new antigens was limited mainly because the bacilli could not be grown in axenic culture. Until then, *M. leprae* had remained an enigma mainly due to its inability to be cultured *in vitro* (Cole et al., 2001). Subsequently, comparison of the genomes and proteomes of *M. tuberculosis* and *M. leprae* revealed that the latter suffers from reduced evolutionary potential. It presented a genome of only 3.3 mega bases compared with 4.4 mega bases of *M. tuberculosis*. This reduction in the *M. leprae* genome has resulted in the elimination of many important metabolic pathways, explaining its intracellular habitat and inability to be cultivated in vitro (Cole et al., 2001).

Since 2000, considerable advances have been made with sequencing of the bacillus DNA. In particular, the 16S rRNA sequence has been used in viability assays, whereas detection of the *M. leprae* mRNAs has been proposed as a promising tool for rapid detection and measurement of viability of the bacilli in the environment (Kurabachew et al., 1998). The major advantage of PCR is its high sensitivity and specificity for detecting DNA from *M. leprae*, without the bacterial culture(Goulart and Goulart, 2008). The technical advances in determining the presence of *M. leprae* in the environment has been complemented by many new findings, such as the elucidation of its 16S rRNA sequence, facilitated by methods such as PCR and Real Time (RT)-PCR (Kadza, 1981; Opromolla, 1997; Abreu et al., 2006).

There were some limitations to the studies discussed in this review, though. First, in the 1980s, detecting acid-alcohol resistant bacilli was not possible due to difficulty in cultivating the bacilli (Salem and Fonseca, 1982; Kadza, 1981). *M. leprae* is deficient in the transport of iron, which is required for cell division, thus making it unlikely that the bacilli can replicate by artificial means (Kato, 1994). The reduction in the *M. leprae* genome might also explain this difficulty (Cole et al., 2011). Secondly, the problem in experimental research in 1995 was the management of refrigeration equipment to preserve the bacilli (Desikan and Sreevatsa, 1995). Exposure to very low temperatures could cause the water to form crystals and harm the bacilli. Moreover, freeze-thaw cycles could also destroy the microorganisms. Thirdly, the small number of counties was an obstacle encountered during research in the state of Ceará (Kerr-Pontes et al., 2006) because of which, the results might not be similar in other parts of the state.

CONCLUSION

This review provides evidence that meteorological factors such as temperature and soil humidity can influence the dynamics of *M. leprae*. The occurrence of this disease is associated with variations in temperature and humidity. However, leprosy cases are registered equally in the rainy season as well as in the dry season, suggesting that *M. leprae* remain viable in various environmental conditions. Therefore, it is very difficult to establish the meteorological pattern to maintain the bacilli in the environment, but there are no doubts about the presence of the bacillus in water, soil as well protected by free-living amoebas. The key aspect in the environment-human transmission appears to be the intensity of exposure to contaminated soil and water that differs between developed and developing countries.

The extensive land area of endemic countries, endemicity in the bordering countries, diversity of biomes, the lack of urban infrastructure, together weather features are possible factors that could influence disease transmission.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

FAPEMAT/PRONEN 2011, research project nº. 477727/2011 is acknowledged.

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Vol. 9(40), pp. 2111-2118, 7 October, 2015
DOI: 10.5897/AJMR2015.7591
Article Number: B7A54ED55927
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Antibacterial *in vitro* assays of new γ-aminoethers and derivatives against Gram-negative and Gram-positive pathogenic bacteria

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Received 24 May, 2015; Accepted 31 August, 2015

A growth inhibition effect against four Gram-negative (*Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae* and *Escherichia coli*) and three Gram-positive (*Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*) pathogenic bacteria was observed for 19 of 20 tested synthetic compounds (that is seven γ-aminoethers, nine γ-aminoalcohols and four allylamines). According to the results, the Gram-negative bacteria were the most susceptible strains toward the tested compounds. In general, the MICs of the active compounds were around 1000 ppm, while the MBCs were around 2000 ppm; however, the allylamine 8a was highlighted for its ability to inhibit *E. faecalis* at the lowest concentration found in this study (MIC = 125 ppm and MBC = 250 ppm).

Key words: Antibacterial activity, γ-aminoether derivatives, minimal inhibitory concentration, minimal bactericidal concentration, Lipinski's rule.

INTRODUCTION

Aminoethers, aminoalcohols and allylamines are related compounds with superior importance not only for their practical applications displayed by themselves but also because they have been found forming part of the structure of synthetic and naturally occurring compounds of diverse practical interest (Cavalluzzi et al., 2007; Huang et al., 2009; Kotland et al., 2011; De Risi et al., 2008; Batra and Nag, 2011; Biava et al. 1999).

Thus, a series of γ-aminoether based selective serotonin (5-HT)-reuptake inhibitor (SSRI) antidepressants (fluoxetine and paroxetine) and the selective norepinephrine (NE)-reuptake inhibitor antidepressants (tomoxetine), have been reported (Pinder and Wieringa, 1993). The naturally occurring aminoalcohol anisomycin (a potent activator of stress-activated protein kinases (JNK/SAPK) and p38 MAP kinase) (Kyriakis et al., 1994) and the phenyl/thienyl-γ-aminoalcohols **1** (direct precursors for the synthesis of fluoxetine, Ar = Ph and duloxetine, Ar = 2-thienyl), have been reported as selective serotonin reuptake inhibitors (Liu et al.,

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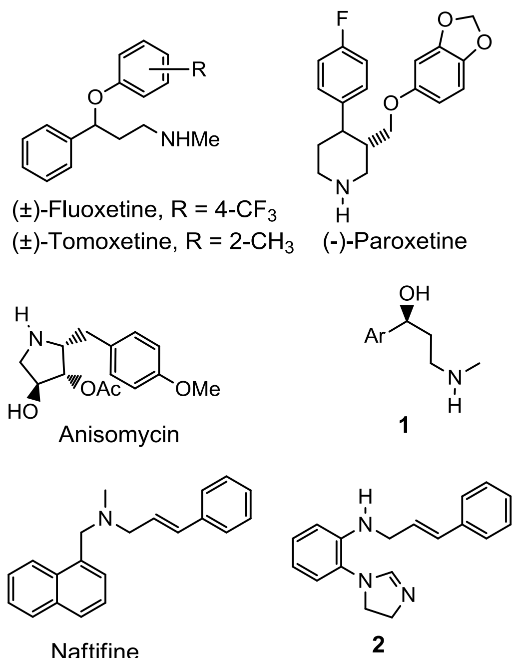


Figure 1. Some aminoethers, aminoalcohols and allylamines of biological interest.

2005). Additionally, several allylamines have been effective in topical treatments for fungal infections of the skin and nails as well as antibacterial (Crawford et al., 2000). Particularly, Naftifine hydrocloride the active ingredient of the commercially available antifungal trademark Naftin® (Jordon et al., 1990) and allylamine **2**, which shows remarkable antagonist activity against mycobacterias and fungal pathogens type *Candida* (Petranyi et al., 1981), are worthy of mention (Figure 1).

Bacteria are champions of evolution, and a few microbes have adapted to a point where they pose serious clinical challenges for humans. In addition, the ever-increasing incidence of antibiotic-resistant infections combined with a weak pipeline of new antibiotics have created a global health crisis against which, novel strategies for enhancing our current antibiotic arsenal are imperatively needed. In response to it, the last decade was characterized by a dramatic increase in the number of antibacterial agents currently under development, which is mainly driven by the urgent problem of multi-drug resistance of bacteria over several commercially available antibiotics (Arias and Murray, 2009; Brynildsen et al., 2013).

In connection with the above and continuing with our current studies on the synthetic utility of benzylamine derivatives (Abonia et al., 2010; Abonia et al., 2013a; Abonia et al., 2013b), herein, we report the preliminary studies on the antibacterial activity of recently synthesized γ-aminoethers **6**, γ-aminoalcohols **7** and allylamines 8 against several Gram-negative and Gram-positive pathogenic bacteria.

MATERIALS AND METHODS

The target compounds **6-8** were obtained by following the multicomponent approaches described in Scheme **1** (Tables 1, 2, 3 and Figure 3). The γ-aminoethers **6** were synthesized, from a four-component procedure, by stirring a mixture of amine **3** (1.0 equiv), polyformaldehyde (1.2 equiv) and the activated alkene **4** (1.0 equiv) in the corresponding alcohol **5** (3 mL) at room temperature. The γ-aminoalcohols **7** were obtained by following the same above procedure but switching alcohols 5 by acetonitrile. Allylamines **8** were obtained either from a three-component reaction in AcOH or by dehydrating the γ-aminoalcohols **7**, previously formed, in refluxing *p*-dioxane mediated by $AlCl_3$ (1 equiv) as catalyst.

Procedures for the antibacterial studies

In order to evaluate the antibacterial activity of compounds **6-8**, the following Gram-negative bacterial strains were used (*Pseudomonas aeruginosa* (ATCC® 15442), *Salmonella typhimurium* (ATCC® 13311), *Klebsiella pneumoniae* (ATCC® 31488), *Escherichia coli* (ATCC® 11229)) and Gram-positive (*Staphylococcus aureus* (ATCC® 25923), *Bacillus cereus* (ATCC® 10876) and *Enterococcus faecalis* (ATCC® 29212)) obtained from American Type Culture Collection.

Bacterial culture conditions

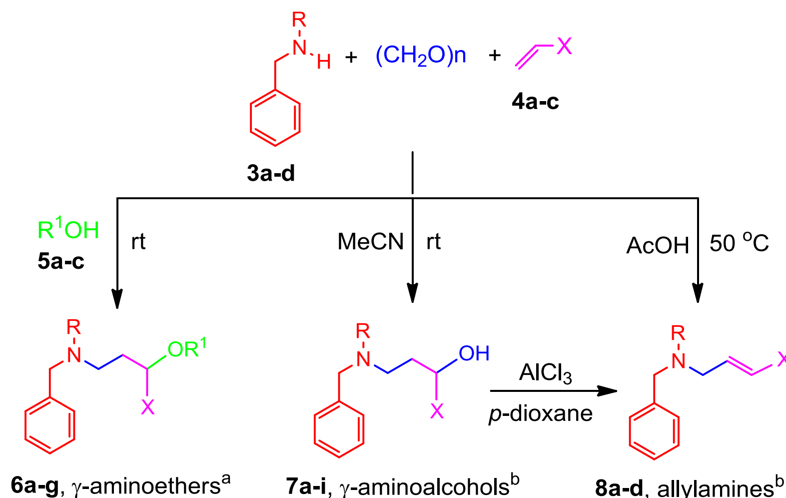
The bacterial strains were previously activated according to the manufacturer instructions and were grown in Muller-Hinton (M-H) broth to 37°C. The time necessary to reach late-exponential phase and bacterial growth were measured by optical density (540 nm), verifying the cell number by plate count. This procedure ensured that the bacterial inoculum was in the same growth phase at a cell concentration in the range of 5 to 45×10^8 CFU/mL.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC is defined as the lowest concentration of antimicrobial agent (µg/mL) that will inhibit the visible growth of a microorganism after 24 h of incubation, and the MBC is the lowest concentration of antimicrobial agent that kills more than 99.9% of the viable organisms after a given incubation time (usually 24 h) (Andrews, 2001).

Broth dilution method

The method proposed by the NCCLS was used (Sisto et al., 2009; Gökçe et al., 2005; Ryan et al., 2002). For each determination a series of 5 test tubes, previously sterilized at 120°C using 15 pounds of pressure for 15 min in a horizontal autoclave, were used and set in the following order: 1.790 mL of (M-H) broth was added to the first test tube and 1 mL into each of the remaining 4 test tubes. Afterwards, 210 µL of the substance to be evaluated, which was previously diluted in DMSO to a concentration of 20000 ppm, was added to the first test tube, obtaining a concentration of 2100 ppm (without inoculum) and a total volume of 2 mL. This solution was mixed using a vortex and 1 mL of it was transferred to the second test tube. This procedure was repeated for the following tubes by transferring 1 mL from the previous tube to the next one in line. Then, each test tube was inoculated with 50 µL of culture of microorganisms in M-H, previously grown to their exponential growth phase. Therefore, the final volume for the five test tubes was 1.05 mL each one (after adding the inoculum), and their final



Scheme 1. General approach for the synthesis of the target γ-aminoethers **6**, γ-aminoalcohols **7** and allylamines **8**. [a](Abonia et al, 2013b). [b]Manuscript in preparation.

Table 1. Minimal inhibitory concentration and minimal bactericidal concentration of the γ-aminoethers **6** evaluated.

Entry	Compound	Inhibited bacteria[a]	MIC (ppm)	MBC (ppm)	Clog P^b	MR (cm^3/mol)[c]	MW[d]	TNA[e]
1	**6a**	*E .coli*	1000	2000	1.96	80.35	276.37	44
		K. pneumoniae	1000	2000				
		S. typhimurium	1000	2000				
2	**6b**	*K. pneumoniae*	1000	2000	3.69	104.85	352.47	54
		S. typhimurium	1000	2000				
3	**6c**	*E. coli*	1000	2000	1.44	53.01	306.40	48
		S. typhimurium	1000	2000				
		E. faecalis	1000	2000				
4	**6d**	*E. coli*	1000	2000	3.69	104.85	249.35	41
		K. pneumoniae	1000	2000				
		S. typhimurium	1000	2000				
5	**6e**	*E. coli*	1000	2000	3.69	104.85	352.47	54
		K. pneumoniae	1000	2000				
		S. typhimurium	500	1000				
6	**6f**	*K. pneumoniae*	1000	2000	4.43	97.92	325.44	51
		S. typhimurium	1000	2000				
		B. cereus	500	1000				
7	**6g**	*E. coli*	1000	2000	3.37	83.03	277.40	47
		P. aeruginosa	1000	2000				
		K. pneumoniae	1000	2000				
		S. typhimurium	1000	2000				

[a]The bacteria names in bold correspond to Gram-positive strains, the remaining are the Gram-negative ones. [b]Calculated log of Partition coefficient. [c]Molar refractivity. [d]Molecular Weight. [e]Total number of atoms.

Table 2. Minimal inhibitory concentration and minimal bactericidal concentration of the γ-aminoalcohols **7** evaluated.

Entry	Compound	Inhibited bacteria[a]	MIC (ppm)	MBC (ppm)	Clog *P*	MR (cm^3/mol)	MW	TNA
8	**7a**	*S. typhimurium*	1000	2000	1.59	75.59	262.35	41
		E. coli	1000	2000				
9	**7b**	***S. aureus***	500	1000	3.33	100.09	338.44	51
		B. cereus	500	1000				
10	**7c**	*P. aeruginosa*	500	1000	2.25	68.87	235.32	38
		K. pneumoniae	500	1000				
		S. typhimurium	500	1000				
		S. aureus	1000	2000				
11	**7d**	*E. coli*	1000	2000	3.65	88.57	297.39	45
		P. aeruginosa	1000	2000				
		K. pneumoniae	2000	2000				
		S. aureus	1000	1000				
12	**7e**	*E. coli*	1000	2000	2.95	121.84	428.52	63
		S. typhimurium	500	1000				
		B. cereus	1000	2000				
13	**7f**	*K. pneumoniae*	1000	2000	1.93	80.39	276.37	44
		B. cereus	1000	2000				
14	**7g**	*E. coli*	1000	2000	2.33	68.67	235.32	38
		K. pneumoniae	500	1000				
		S. typhimurium	500	1000				
		E. faecalis	1000	2000				
		B. cereus	500	1000				
15	**7h**	*E. coli*	250	500	4.06	93.17	311.42	48
		P. aeruginosa	1000	2000				
		K. pneumoniae	250	500				
		S. typhimurium	250	500				
16	**7i**	*K. pneumoniae*	1000	2000	2.73	130.01	472.53	66
		S. typhimurium	1000	2000				

[a]The bacteria names in bold correspond to Gram-positive strains, the remaining are the Gram-negative ones.

concentrations were 2000, 1000, 500, 250 and 125 ppm, respectively. A test tube containing 1 mL of broth culture, without inoculum, was included as negative control. A test tube containing only broth culture and the bacterial inoculum was set as the positive control. All the above was performed in triplicate and incubated at 35°C for 24 h.

Reading of results

MIC results were reported taking into account the immediately previous test tube to the one which presented growth of microorganisms, determined by turbidity (Figure 2), or growth on plate. This last procedure was carried out when the substances caused an initial turbidity after they were added to the growth medium.

Minimal bactericidal concentration test (MBC)

From the test tubes that did not show apparent bacterial growth in the MIC experiments, 0.1 mL of solution was taken and spread in Petri dishes with M-H agar and incubated for 24 h at 37°C. Taking into account the test tube from which the inoculum was taken, the concentration of antimicrobial agent necessary for inhibiting bacterial growth was determined.

Experimental design

The experiment was carried out using a two factor design in which the first factor corresponded to the number of substances used (20) and the second, the different concentrations (5) to which the bacterial strains were exposed in this study and the experiments

Table 3. Minimal inhibitory concentration and minimal bactericidal concentration of the allylamines **8** evaluated.

Entry	Compound	Inhibited bacteria[a]	MIC (ppm)	MBC (ppm)	Clog *P*	MR (cm^3/mol)	MW	TNA
17	**8a**	***E. faecalis***	125	250	3.53	98.92	320.43	48
		B. cereus	1000	2000				
18	**8b**	*E. coli*	1000	2000	1.80	74.43	244.33	38
		P. aeruginosa	1000	2000				
		K. pneumoniae	1000	2000				
		S. typhimurium	2000	2000				
		S. aereus	1000	2000				
		B. cereus	500	1000				
19	**8c**	*E. coli*	1000	2000	2.14	79.23	258.36	41
		S. typhimurium	1000	2000				
20	**8d**	*All bacterial strains*	No inhibition	No inhibition	3.15	120.67	410.51	60

[a]The bacteria names in bold correspond to Gram-positive strains, the remaining are the Gram-negative ones.

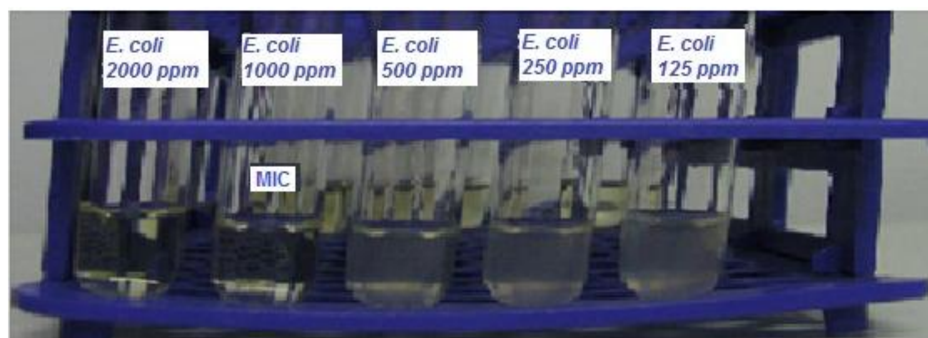


Figure 2. A representative picture for the MIC determination against Gram-negative bacteria.

were carried out by triplicate. Since the results obtained in the MIC and MBC tests were qualitative (inhibition, no inhibition), the responses corresponded to binary variables, and in addition, all the repetitions had identical results. It can be deduced that there was no observed variability in the different treatments because all the results were the same for all the repetitions; hence, it was not possible to perform a parametric inferential analysis. This fact is because, according to the method used in this work, counting of cells or colony forming units on bacterial plates, which could have some variability, is unnecessary and was not performed.

RESULTS AND DISCUSSION

Figure 3 summarizes the structure of the obtained compounds for antibacterial evaluation.

All synthesized compounds have the capability to form hydrogen bonds due to the nitrogen atom present in their structures. This feature could make it possible for them to bind to the molecules of the bacterial structure, by either allowing them to bind to the wall or external membrane and to be transported within the bacteria. A growth inhibition effect was observed for 19 of 20 tested compounds (that is γ-aminoethers **6a-g**, γ-aminoalcohols **7a-i** and allylamines **8a-c**), with the exception of the allylamine **8d** (Tables 1 to 3). In general, the minimal inhibitory concentrations (MICs) of the active substances were around 1000 ppm, while the MBCs were around 2000 ppm.

Among γ-aminoethers **6** (Table 1), all evaluated substances affected *S. typhimurium*, continued by *K. pneumoniae*, which was inhibited by six of the seven compounds with a MIC of 1000 ppm and a MBC of 2000 ppm. Compound **6g** affected all Gram-negative bacteria

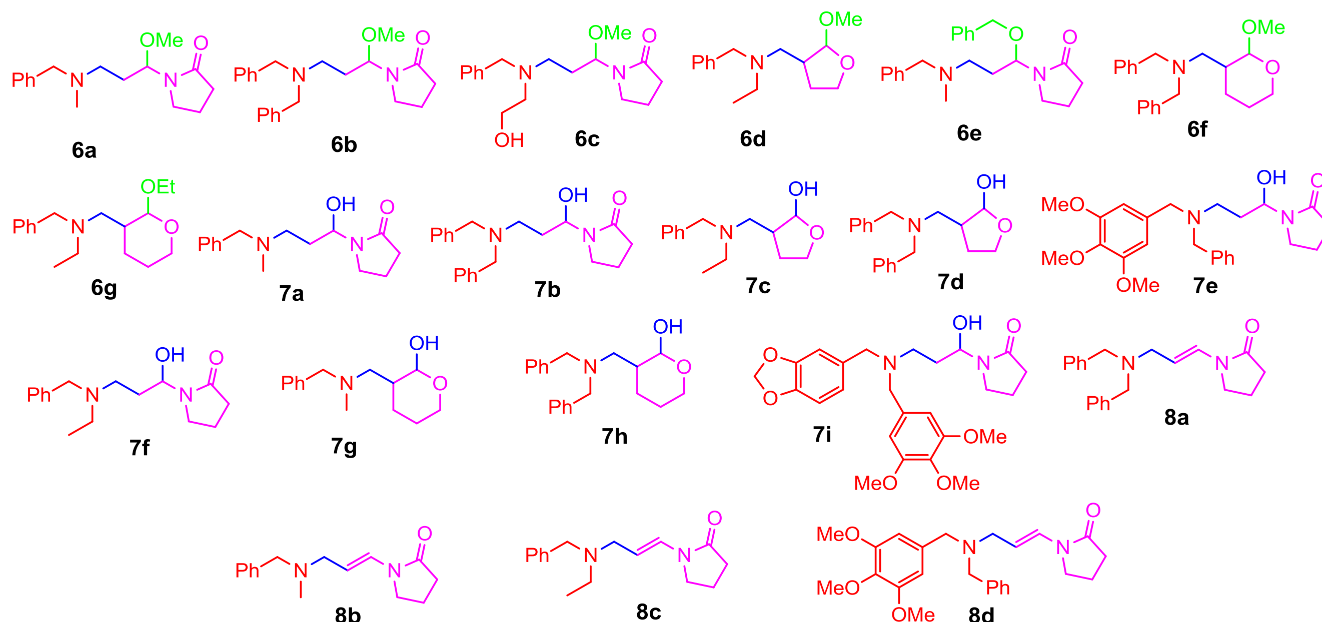


Figure 3. Chemset of the obtained γ-aminoethers **6**, γ-aminoalcohols **7** and allylamines **8** used for antibacterial tests.

with MIC of 1000 ppm and MBC of 2000 ppm. Compound **6f** was comparatively the most outstanding substance of this group because it had the relatively lowest MIC and MBC values. In Gram-positive bacteria, this group of compounds was the least effective, having presented bactericidal activity in only two (that is **6c** and **6f**) of the seven compounds which affected *E. faecalis* and *B. cereus*, respectively.

In general, γ-aminoethers **6** were noted for their bactericidal action against Gram-negative bacteria, because out of the seven compounds tested, five of them (**6a**, **6d**, **6e**, **6f** and **6g**) exhibited antibiosis against this family of microorganisms. Moreover, the most susceptible strain toward the γ-aminoethers **6** (*S. typhimurimum*), which was inhibited by all compounds **6**, showed that this bacterium was particularly susceptible to the benzyl groups present in such structures.

On the other hand, all nine evaluated γ-aminoalcohols **7** showed inhibitory effects on the studied bacteria, being the greatest inhibition against Gram-negative bacteria. Among them *K. pneumoniae* and *S. typhimurium* were susceptible to six of the nine compounds **7**, while *E. coli* was sensible to five of them (Table 2). Compounds **7g** and **7h** are highlighted, the first one, for inhibiting a greater number of bacterial strains Gram-negative as well as Gram-positive, and the second one, for presenting the lowest MICs (250 ppm) and MBCs (500 ppm) values of this group, affecting the growing of all the studied Gram-negative bacteria, although it did not affect any of the Gram-positives. Gram-positive bacteria showed a higher resistance to these types of compounds; *B. cereus* was affected by four compounds, *S. aureus* by three and *E. faecalis* by only one of them (Table 2).

It was also observed, that some functional groups in **7** determined the biological activity of these molecules. That is how a different behavior was observed for each of the nine tested compounds **7** when the substituents were pyran, pyrrolidone or furan. Although, this group of compounds was the most active, since all of them showed bactericidal effect against at least one strain of the study, apparently, the presence of pyran and benzyl groups simultaneously in the molecule was the better combination for the widest spectrum of activities and lowest MICs and MBCs values as shown by compounds **7g** and **7h** (Table 2).

With regard to allylamines **8**, from the four compounds that were evaluated (**8a-d**), three of them showed any type of activity (Table 3). Compound **8b** achieved growth inhibition for six of the seven evaluated bacterial strains, while, compound **8a** presented the lowest MIC (125 ppm) as well as the lowest MBC (250 ppm) from all studied compounds by negatively affecting *E. faecalis*, although it did not show any effect on the Gram-negative strains. Particularly, allylamine **8d** was the unique compound which did not present any antibiosis against any bacteria in this study.

Moreover, for allylamines, when R was a benzyl group (i.e. **8a**), only Gram-positive bacteria were affected; but when it was a methyl (**8b**), the spectrum of action was broadened to include the Gram-negative bacteria also (Table 3). In contrast, the inactivity observed for compound **8d** (structurally analogue to **8a**) should be associated with the presence of the methoxyl groups in the R substituent, which could not contribute to its

lipophilicity and hence to its bactericidal activity.

It is known that the Lipinski's rule ("the rule of 5") is a qualitative rule published in 1997 based on parameters such as log *P* (Partition coefficient), molar refractivity (MR), molecular weight (MW), total number of atoms (TNA) and number of donors/acceptors hydrogen bonding to predict the lipophilicity of a small molecule associate with its poor or good permeation/absorption capability to cross the cell wall and for instance determine its activity (Lipinski et al., 1997; Leo et al., 1971). Subsequently, Ghose et al. (1999) inspired by Lipinski's rule, performed a qualitative and quantitative characterization of known drugs based on Comprehensive Medicinal Chemistry (CMC) databases, which included some central nervous system active drugs and cardiovascular, cancer, inflammation, and infection disease states (including several antibacterials). The study afforded average values for the aforementioned parameters (calculated log *P* (Clog *P*) = 2.52, MR = 97, MW = 357, and TNA = 48) for the different classes of drug molecules studied. Additionally, benzene was the most abundant structural unit found in such drug database (Ghose et al., 1999).

Tables 1, 2 and 3 show the values of Clog *P,* molar refractivity, molecular weight and total number of atoms determined for all twenty compounds in our study (Calculated octanol-water, 2014). A raw comparative analysis suggests compounds **6f**, **7h** and **8a** as relatively more active in their corresponding series because of their comparatively lower values of MIC and MBC. The Clog *P*, MR, MW and TNA values were 4.43, 97.92, 325.44 and 51; 4.06, 93.17, 311.42 and 48 and 3.53, 98.92, 320.43 and 48 for compounds **6f**, **7h** and **8a** respectively. Interestingly, several values of the above four parameters, match better with some of the average values (2.52, 97, 357 and 48) determined by Ghose et al. (1999) than those for the remaining compounds of the studied series. This means that there is relative agreement between the Lipinski´s rule parameters and the activity found for the more active compounds **6f**, **7h** and **8a** of the three series **6**, **7** and **8** respectively. Moreover, all three compounds possess the dibenzylamino moiety (two free phenyl groups content) which are in agreement with findings by Ghose et al. (1999).

Finally, it is worth mentioning that *P. aeruginosa* is one of the leading Gram-negative organisms tightly associated with nosocomial infections and their consequences for immunocompromised patients. The increasing frequency of multi-drug-resistant *P. aeruginosa* (MDRPA) strains confirms that efficacious antimicrobial options for their treatment are currently limited (Obritsch et al., 2005). In this sense, the fact that five of the evaluated compounds (**6g**, **7c**, **7d**, **7h** and **8b**) were active (although in moderate strength, MIC´s = 500-1000 ppm), it is a remarkable finding because of the current urgency for new active drugs against these kind of pathogens. Our modest results could be a starting point for this purpose.

Conclusion

In summary, the evaluated substances showed differential antibacterial activity between both strains, showing that the Gram-negative bacteria were the most susceptible ones. Indeed, *S. typhimurium*, *K. pneumoniae*, *E. coli* and *P. aeruginosa* were sensible to 15, 13, 12 and 5 of the evaluated compounds, respectively. Meanwhile, Gram-positive bacteria were more resistant, according to the observed behavior in *B. cereus*, *S. aureus* and *E. faecalis*. They were affected by 7, 4 and 3 of the evaluated compounds respectively, which produced a negative effect on their growth. The allylamine **8a** is highlighted for its ability to inhibit *E. faecalis* at the lowest concentration found in this study, with a MIC of 125 ppm and a MBC of 250 ppm. The four parameter values (that is, Clog *P*, molar refractivity, molecular weight and total number of atoms) for the more active compounds **6f**, **7h** and **8a**, were in relative agreement with the Lipinski´s rule and the qualitative/quantitative characterization of known drugs database performed by Ghose and co-workers.

Although it was not possible to establish a rigorous activity-structure relationship due to the relative high MIC and MBC values, certainly, it can be assumed that some functional groups in compounds **6**, **7** and **8** could be responsible for their biological activities.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

Authors thank COLCIENCIAS and Universidad del Valle for the financial support.

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Vol. 9(40), pp. 2119-2134, 7 October, 2015
DOI: 10.5897/AJMR2015.7453
Article Number: CAA2B0A55930
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of vegetation and seasonality on bacterial communities in Amazonian dark earth and adjacent soils

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Received 27 February, 2015; Accepted 10 August, 2015

Amazonian Dark Earth (ADE) in the Brazilian Amazon is the main evidence left by pre-Columbian indigenous populations indicating that infertile soils can be transformed into highly fertile ground. Changes in vegetation cover and seasonality are likely to influence microbial communities; however, little is known about these effects on ADE. Therefore, this study compared the effects of two land use systems in ADE and adjacent soil (ADJ) during the rainy and dry seasons using biochemical and molecular tools. Bacterial community function was determined by community level physiological profile (CLPP), bacterial community structure by terminal restriction length polymorphism (T-RFLP), and bacterial community composition by pyrosequencing of the V4 16S rRNA gene region. Our results show that the community structure is highly affected by vegetation, in both, ADE and ADJ soils. Regarding community function, Average Well Color Development (from Biolog substrates) were higher in ADE than ADJ during the rainy season and kept the same pattern of substrate utilization during the dry season and finally, community composition showed to be influenced even at the level of family, mostly by soil type rather than vegetation. Collectively, our study provides insights into processes affecting the bacterial community assemblages in both, ADE and adjacent soils.

Key words: Amazonian soils, vegetation type, seasonality, soil bacteria.

INTRODUCTION

Most of the upland Amazon rainforest is located on heavily weathered and nutrient-poor soils. Their productivity depends on vegetation diversity and also relies on the efficient recycling of organic matter (Sanchez et al., 1982). Slash-and-burn agriculture is a typical smallholder land use system in the Amazon region. The release of nutrient-rich ashes leads to an increase in soil pH and cation contents of the surface soil layer, consequently providing new nutrient input (Hölscher et al., 1997). However, after continuous use for

cropping, there is a gradual decrease in soil fertility (Sanchez et al., 1982); another factor is nutrient losses due to the burn, harvest, and leaching during the process of slash-and-burn agriculture (Hölscher et al., 1997).

Concerning the same region, the existence of scattered patches of fertile black soils know as Amazonian Dark Earth (ADE) (locally called *Terra Preta de Índio*) is the main evidence left by pre-Columbian indigenous populations indicating that poor soil can be transformed into highly fertile ground. Analyses of this anthropogenic soil have shown that they present high levels of stable organic matter and chemical nutrients, such as carbon, phosphorous, calcium and manganese (Lehmann et al., 2003). Moreover, the anthropic horizon of ADE shows high resilience to soil management and remarkable soil physical qualities, such as good soil aggregation and high porosity in comparison to the surrounding soils (Teixeira and Martins, 2003). It is believed that these elements were added to the soils through human depositional activity and prehistoric semi-intensive or intensive agriculture (Denevan, 1996). For these reasons, anthropogenic ADE is frequently cultivated by traditional smallholders for subsistence farming.

In spite of the unique properties of ADE, little is known about the effects of modern agricultural practices, current land use, and seasonality effects on these anthrosols. Furthermore, different types of aboveground vegetation are known to influence soil bacterial communities (Mitchell et al., 2010; Chaparro et al., 2012). There is also growing concern that current climate change may cause a large “dieback”or degradation of Amazonian rainforest with a higher probability of intensified dry seasons (Malhi et al., 2009). This, in turn, will influence soil microbial communities which mostly regulate ecosystem processes (Neher, 1999). Few studies have characterized the bacterial community composition and distribution in different ADE sites (O´Neill et al., 2009; Grossman et al., 2010; Navarrete et al., 2010). Recently, using the DNA pyrosequencing technology, Taketani et al. (2013) observed that vegetation cover had an effect over the bacterial community structure independent of soil type and in the same sites of the present study.

Therefore, it is important to further assess ADE microbial communities to identify possible shifts in these communities that may influence soil fertility and quality. One way to assess changes in soil function is the use of Biolog ecoplates to generate a community-level physiological profile (CLPP) of mixed aerobic heterotrophic bacteria (Garland and Mills, 1991). Despite the methodological implications of BIOLOG ecoplates, the method was successful used to detect differences in microbial communities in soil such as Arctic tundra soils (Campbell et al., 2010) and wetlands under different land management regimes (Doutorelo et al., 2010). The molecular toolbox [group-specific-PCR; Denaturing Gradient Gel Electrophoresis (DGGE); Terminal Restriction Fragment Length Polymorphism (T-RFLP)] has also been successfully used to describe changes in microbial community structure in tropical forest soils (Jesus et al., 2009) and agricultural soils (Enwall et al., 2007) and DNA pyrosequencing technology has proven to be a powerful tool for rapid and sensitive investigations into complex microbial communities.

Here we investigated the bacterial community function, structure and composition at finer taxonomic level in ADE (Hortic Anthrosol) and the adjacent soil (Haplic Acrisol, ADJ) under different vegetation types and seasons at the Caldeirão Experimental Research Station in the Brazilian Central Amazon. This study combined CLPP, T-RFLP and pyrosequencing technology to test the hypothesis that aboveground plant diversity and seasonal effects might differentially influence the ADE and ADJ inhabiting bacterial communities. In addition, we provide correlational insights relating the relative abundance of bacterial families and genera in these soils to the differences between the soil chemical properties detected among sites.

MATERIALS AND METHODS

Study sites and soil sampling

The studied sites were located in the Caldeirão Experimental Research Station of Embrapa Amazônia Ocidental in Iranduba County in the Brazilian Central Amazon (03°26’00”S, 60°23’00”W). Four different sites were chosen based on the presence of pre-historic anthropic soil horizons (Hortic Anthrosols) referred to as ADE, along with the adjacent soils without an anthropic horizon (Haplic Acrisol, ADJ) according to the World Reference Base for Soil Resources (FAO, 1998). At both sites, the vegetation cover types were a 35-year-old secondary forest (SF) and a 5-year-old manioc (*Manihot esculenta*) plantation. The soil samples were collected during the rainy season (January 2009) with mean monthly rainfall of approximately 400 mm, and the dry season (August 2009) with mean monthly rainfall of approximately 30 mm (http://clima1.cptec.inpe.br/~rclima1/monitoramento_brasil.shtml).
At each site, the sample plot was determined by choosing a random point and from this reference point, three points 5 m apart were chosen for the collection of intact soil cores 5 cm in diameter and 15 cm in length. Soil samples were collected using sterile techniques and transported (< 24 h) in an isolated box on dry ice for DNA extraction and on ice packs for physiological and microbial biomass measurements at CENA in Piracicaba (SP, Brazil). Total microbial biomass measurement was performed at Embrapa Soybean (Londrina, Brazil), and chemical analysis at Embrapa Amazônia Ocidental in Manaus, Brazil.

Determination of soil chemical properties

Soil samples were analyzed in triplicate for pH (H_2O, 1:1), soil

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extractable Al, Ca, and Mg (1 M KCl), soil extractable P and K (double acid solution of 0.025 M sulfuric acid and 0.05 M hydrochloric acid Mehlich 1), soil C (Walkely-Black method) and effective cation exchange capacity (sum of all base cations plus exchangeable Al and H). For more details on the methods used for such measurements (Embrapa, 1998). The soil moisture was determined after drying the samples overnight at 105°C.

Soil microbial biomass carbon (MBC) was estimated following the fumigation-extraction method (Vance et al., 1987) and soil microbial biomass nitrogen (MBN) was assessed by the method of Brookes et al. (1985), both slightly modified by Hungria et al. (2009). For both measurements, triplicates were used from each site (n = 9). MBC measurements were based on the difference between organic C extracted with 0.5 M K_2SO_4 (Bartlett and Ross, 1988) from chloroform fumigated and unfumigated soil samples (Vance et al., 1987), using a correction factor of 0.41 as recommended for tropical soils (Feigl et al., 1995). MBN was determined by the difference between extractable N in fumigated and unfumigated samples using a correction factor of 0.54 (Brookes et al., 1985).

Biolog functional analysis

Microbial community level physiological profiles (CLPP) were assessed using Biolog Ecoplates® (Biolog, Hayward, CA, USA) which contained three replicate wells of 31 carbon sources and a water blank (Insam, 1997). Measurements were performed for each soil sample collected from the three points of each site with three replicates per carbon substrate (n = 9). Inoculation density was previously estimated by counting colony forming units on nutrient agar medium at 25°C for 48 h. Each soil suspension was inoculated into Biolog Ecoplates (120 µL per well) which were incubated at 28°C and were read after 12 h, then every 24 h for seven days using an ELISA microplate reader at 590 nm. The generated Biolog ecoplate data were transformed by dividing the raw values by the respective average well color development (AWCD) values (Garland and Mills, 1991). The corrected values were used to evaluate average heterotrophic metabolism and to estimate kinetic parameters as proposed by Lindström et al. (1998): $AWCD = K / [1 + e^{-r(t-s)}]$, where K (asymptote) is the maximum degree of color development, R (degradation rate) is the exponential rate of AWCD change (h^{-1}), t is the time of following inoculation of the plates (h), and S is the time when the mid-point of the exponential portion of the curve (that is when Y=K/2) has been reached (h).

DNA extraction, T-RFLP and 454-pyrosequencing

Soil DNA was extracted in triplicate for each sample using the MoBio PowerSoil DNA extraction kit according to the manufacturer's instructions (MoBio Laboratories, USA). The purity and quantity of the extracted DNA were determined by UV-spectrophotometry at 260 and 280 nm (NanoDrop® ND-1000 UV/vis-spectrophotometer, Peqlab Biotechnologie GmbH, Erlangen, Germany). The obtained DNAs were further stored at −20°C.

T-RFLP analysis was performed with the primer set 27F-FAM-labeled (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), used to amplify the near-full 16S rRNA gene (Lane, 1991). Each PCR amplification was performed in triplicate (*n* = 9) in 25 µL reactions containing 2.5 µL 10x reaction buffer (Invitrogen, Carlsbad, CA), 3 mM $MgCl_2$, 0.2 mM of each dNTP (Eppendorf, Germany), 0.1 mM BSA (New England Biolabs, USA), 0.25 mM forward-labeled primer 27F, 0.25 mM reverse primer 1492R, 1 U of Platinum Taq DNA Polymerase (Invitrogen, USA), and 2 ng of template DNA. Cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 59°C for 45 s, 72°C for 1 min, and a final extension step of 72°C for 15 min. Obtained products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) and digested at 37°C for 3 h with the endonuclease MspI (Invitrogen, USA). DNA was precipitated using isopropanol (Sambrook and Russell, 2001) and resuspended in 9.8 µL of deionized formamide and 0.2 µL of GeneScan-500 ROX internal size standard (Applied Biosystems, USA), then denatured at 94°C for 5 min. Terminal Restriction Fragments (TRFs) were analyzed using an ABI PRISM 3100 genetic sequencer (Applied Biosystems, USA).

Partial bacterial 16S rRNA gene sequences were amplified for pyrosequencing using the following primers to target the V4 region (fragment length of 270-300 bp) of the 16S rRNA gene at corresponding *Escherichia coli* positions 563 and 802: primers 563F and 802R (Sul et al., 2011) containing the Roche 454 pyrosequecing adaptors and barcodes of 8 bp (attached to the forward primers). Each PCR reaction mixture contained 1x reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 10 mg mL^{-1} of BSA, 0.2 µM of each primer, 1 U of FastStart high-fidelity PCR system enzyme blend (Roche Applied Sciences, IN, USA), and 4 ng of DNA template. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 45 s, and 72°C for 1 min, and a final extension step of 72°C for 4 min. PCR products were separated by agarose gel electrophoresis and the products with the expected size (ca. 270-300 bp) were excised and purified using the Qiagen gel extraction kit (Qiagen, CA, USA), followed by a second purification with the Qiagen PCR purification kit (Qiagen, CA, USA). Sequencing was performed on the GS FLX sequencer (454 Life Sciences, CT, USA) at the Michigan State University Research Technology Support Facility. The dominant phyla and class composition of the bacterial communities from the same sites of this study was previously reported (Taketani et al., 2013). Here, we incorporated such dataset to gain insights into a deepest taxonomical resolution of such effects.

Soil chemical properties, microbial biomass, and Biolog data analysis

Variance analyses of soil chemical properties were tested separately for land use and season by ANOVA. Results showing significant overall changes were subjected to Tukey's post-hoc test with significance set at $P < 0.005$. The kinetic parameters were submitted to analysis of variance (ANOVA) and differing pairs were identified with post hoc Tukey test ($P < 0.05$). These results were also correlated with soil chemical properties and microbial biomass using the Spearman correlation coefficient. Statistical analyses were carried out using STATISTICA version 10 (StatSoft, USA).

T-RFLP data analysis

T-RFLP data were analyzed using Peak Scanner software v1.0 (Applied Biosystems). TRFs smaller than 50 bp and larger than 800 bp were excluded from the analysis. True peaks were determined using T-REX online software according to Abdo et al. (2006) (http://trex.biohpc.org, last updated on 2010/03/01). TRF sizes were rounded to the nearest integer and peak heights were relativized to account for uncontrolled differences in the quantity of DNA between samples (Culman et al., 2009). Normalized peak heights were used to calculate the relative abundance of TRFs. Statistical analysis of T-RFLP data were performed on square-root transformed data to obtain homogeneity of variances. Multivariate analysis of the T-RFLP fingerprints from all sites was performed using multidimensional scaling (MDS) based on Bray-Curtis similarity matrices. Permutational ANOVA (PERMANOVA) was used to verify significant differences between samples from all sites and seasons (Anderson, 2001). The influence of soil properties on the bacterial community structure was assessed using BEST analysis (BIOENV procedure), which selects the soil properties that may

Table 1. Selected soil properties of Amazonian Dark Earth and adjacent soil (Haplic Acrisol) under secondary forest and manioc plantation during the rainy and dry seasons.

Soil properties	Amazonian Dark Earth				Adjacent Soil			
	Secondary forest		Manioc plantation		Secondary forest		Manioc plantation	
	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry
pH	5.40[a]	5.25a	5.46a	5.33a	3.63b	3.67b	3.68b	3.73b
Soil C (g kg^{-1})	32.35a	28.17a	28.44a	26.47a	30.47aA	18.68aB	17.62b	16.15b
P (mg dm^{-3})	140aA	83aB	174a	205a	9b	4b	6b	4b
Ca ($cmol_c$ dm^{-3})	9.05aA	3.93aB	8.68aA	3.93aB	0.89b	0.24b	0.16b	0.11b
Mg ($cmol_c$ dm^{-3})	1.43aA	0.86aB	1.53a	1.11a	0.31b	0.11b	0.10b	0.06b
Al ($cmol_c$ dm^{-3})	0.01a	0.03a	0.01a	0.02a	2.06bA	1.52bB	1.72b	1.49b
CEC† ($cmol_c$ dm^{-3})	10.64aA	4.90aB	10.37aA	5.13aB	3.42b	1.95b	2.07b	1.74b
MBC (mg kg^{-1})	656.97aA	431.00aB	372.53b	346.43ab	378.53b	452.93a	248.33c	232.73b
MBN (mg kg^{-1})	51.93a	63.47a	20.90bA	15.33bB	19.37bA	51.33aB	12.83b	13.00b
SMC (%)	41.8aA	23.1aB	24.0bA	16.2bB	40.0aA	12.7bcB	37.7aA	12.1cB

[a]Abbreviations: CEC, cation exchange capacity; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SMC, soil moisture content. [b]Means separately for eADJ season within a column followed by the same lower case letter are not significantly different ($P < 0.05$, Tukey post hoc).[c]Significant differences between seasons are followed by different upper case letter ($P < 0.05$, Tukey pos hoc).

explain biotic patterns (Clarke, 1993). All multivariate statistical analyses aforementioned were performed with PRIMER 6 software and the PERMANOVA add-on (Clarke and Gorley, 2006; Anderson et al., 2008).

Pyrosequencing data analysis

The resulting sequence reads were screened to remove sequences that contained any errors in the forward primer and barcode regions, ambiguities, and sequences shorter than 150 bp using the RDP Pyrosequencing Initial Process Tool (Cole et al., 2009). Chimeric sequences were identified by the Chimera Check program in the RDP pipeline (http://www.rdp.cmc.msu.edu). Quality trimmed sequences were aligned using the RDP pyrosequencing function *Aligner* and clustered with default parameters of the RDP function *Clustering*. The resulting aligments were manually checked and corrected if necessary. The resulting clusters were used to construct rarefaction curves at a dissimilarity value of 3% and were subsequently phylogenetically classified using the RDP Classifier (Wang et al., 2007). Distance matrices were constructed using the *dist.seqs* function and LIBSHUFF comparisons were made between the four studied sites using MOTHUR software (Schloss et al., 2009).

RESULTS

Soil properties and microbial biomass

The results of the different soil properties measured in ADE and ADJ under secondary forest (SF) and manioc plantation (M) during the rainy and dry seasons are presented in Table 1. Soil chemical properties of ADE-SF were chemically similar to ADE-M during both seasons. ADJ-SF and ADJ-M chemical properties were also very similar with the exception of soil organic carbon (SOC), which was significantly higher in ADJ-SF. ADE showed higher soil pH independent of vegetation comparatively to ADJ. As expected, in contrast to ADJ, ADE showed higher CEC, Ca, Mg, and P, indicating the high fertility of these anthropic soil horizons. Particularly, ADE had significant higher exchangeable bases (Ca, Mg) at both sites compared to ADJ. Decreases in the Ca content were observed during the dry season in ADE-SF (57%) and ADE-CP sites (55%). Similarly, there was a significant decrease (40%) in the Mg content, but this was only observed in ADE-SF. Seasonal changes in CEC were also observed in ADE for both sites with a significant decrease during the dry season. For ADJ, seasonal changes influenced only the contents of SOC and SOM under SF.

Microbial biomass carbon (MBC) was higher in ADE-SF compared to ADE-M, which presented similar MBC values as ADJ-SF during the rainy season. ADJ-M showed a decrease in MBC and MBN values for both seasons. Furthermore,

seasonality affected MBC in ADE-SF with a 34% decrease along with a 27% reduction in MBN for ADE during the dry season. For ADJ-SF, there was a significant increase in MBC and MBN from the rainy to the dry season. On the other hand, ADJ-M presented a significant decrease in MBC contents from the rainy season to the dry season. Soil moisture content decreased by 45-68% from the rainy to the dry season.

Bacterial community function

Average Well Color Development (AWCD) data represented by the average utilization intensity of 31 carbon substrates (during the evaluation period) are shown in Figure 1. The AWCD of plates inoculated with all studied soil samples increased rapidly after 30 h in both seasons, with the exception of ACH-SF-Rainy. In the rainy season, AWCD varied among the different soil types with higher overall AWCD values in ADE compared to the ACH soils. Differences due to vegetation type were observed only for the ACH soil samples with the lowest activity in ACH-SF. Nevertheless, there were no changes in AWCD for all soils in the dry season. Microbial utilization patterns of specific substrate groups are presented in Figure 2. Differences in microbial utilization patterns were observed only during the rainy season. The microbial utilization of carbohydrates was higher in ADJ-SF during the rainy season. Furthermore, the ADJ-SF presented lower microbial utilization of carboxylic and acetic acids, amino acids and amines when compared to the other sites.

Bacterial community structure

T-RFLP data analysis by multidimensional scaling (MDS) showed clearly differences between community structures in ADE and ADJ, and distinct clusters were formed according to vegetation type and sampling period (Figure 3). These results were further statistically confirmed by PERMANOVA, showing a significant effect of both, vegetation (SF and CP) and seasonality (rainy-R; dry-D) ($P = 0.002$). The BIO-ENV routine was used to determine which set of variables (environmental and microbial biomass) mostly explained the biological patterns observed in the T-RFLP analysis. The results indicate Al, Ca, P, pH, and SMC (Rho = 0.911; $P < 0.01$) as major drivers of community structure in the rainy season. For the dry season, Al, MBN and pH (Rho = 0.877; $P < 0.01$) were the major variables explaining the observed distribution.

Bacterial community composition

The pyrosequencing-based analysis of the V4 region of 16S rRNA was previously used to assess the bacterial community of ADE and ADJ (Taketani et al. 2013). It was shown that the most abundant phyla in all sites were Actinobacteria, Acidobacteria, Verrucomicrobia and Proteobacteria, represented by approximately 70% of the total number of sequences. However, at the class level, community composition showed differences between ADE and ADJ and, also, an effect of vegetation type was observed. In this sense we here use the same dataset to investigate these effects at a deepest taxonomic level.

Classification of sequences at the family and genus levels showed differences in their relative abundances according to the soil and vegetation type (Tables 2 and 3). The ADE soil was dominated by Gaiellaceae, Gemmataceae and Syntrophobacteraceae. In the ADJ soil, Acidobacteriaceae, Acetobacteraceae, Alicyclobacillaceae, Burkholderiaceae, Caulobacteraceae, Conexibacteraceae, Sinobacteraceae, Solibacteraceae and Xanthomonadaceae were the most abundant. Relative abundance of Hyphomicrobiaceae was higher in both soils under secondary forest. Moreover, higher bacterial family abundance in both soils under manioc plantation included Gemmataceae, Thermogemmatisporaceae and Oxalobacteraceae. At the genus level, the most dominant genera were *Alicyclobacillus*, *Bradyrhizobium*, *Candidatus solibacter* and *Rhodoplanes*. Among the most abundant genera under secondary forest were *Burkholderia* and *Rhodoplanes*. The genera *Luteibacter* and *Salinispora* were only observed in the ADJ soils. The relative abundance of bacterial families and genera lower than 1% also confirmed differences between ADE and ADJ soils (Tables S1 and S2).

We analyzed the relationship between bacterial family relative abundance and soil properties using Spearman correlation (Table 4). Most of the selected bacterial families were negatively correlated with soil properties typically found in higher amounts in ADE soils, indicating that ADJ soil properties may favor the higher abundance of these bacterial groups. Gaiellaceae, Gemmataceae and Syntrophobacteriaceae presented positive correlation with ADE soil properties and negative correlation with Al. In specific, the relative abundance of Gaiellaceae showed strong positive correlation with Ca, Mg and CEC, while the abundance of Syntrophobacteriaceae was positively correlated with P.

DISCUSSION

Temporal variability in soil properties

Losses of SOC and SOM by the conversion of native forest to agricultural use in the Brazilian Amazon are well known (Fearnside and Barbosa, 1998). This is in agreement with the results obtained in the ADJ soil samples, which showed a significant decrease in SOC and SOM after the conversion of secondary forest (SF) to a manioc plantation (M). However, SOC and SOM in

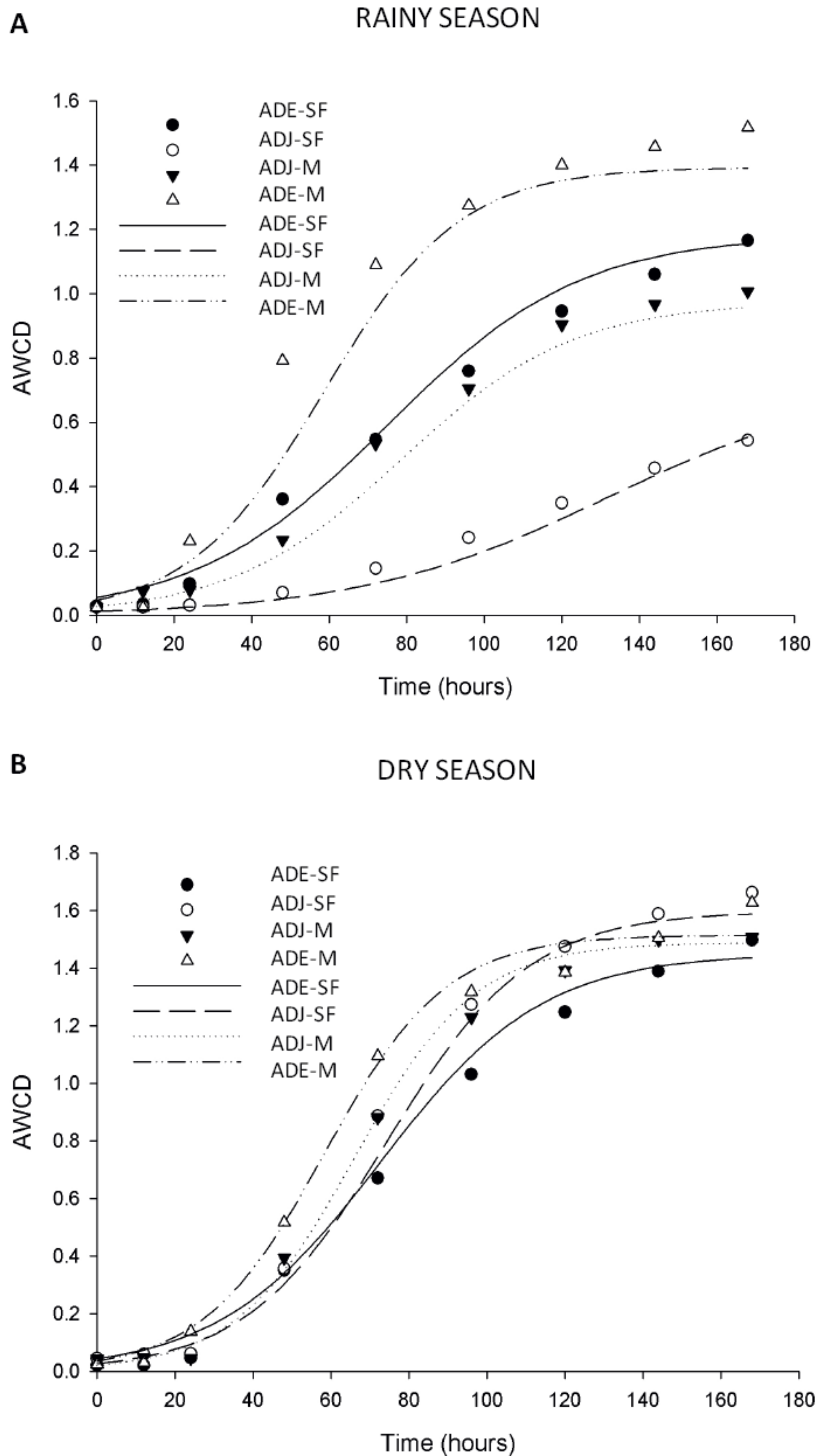

Figure 1. Average well color development (AWCD) of community-level physiological profiles (CLPP) of Amazonian Dark Earth (ADE) and adjacent soil (ADJ) under secondary forest (SF) and manioc plantation (CP) during the rainy (A) and dry (B) seasons. The lines represent the fitted equations and the dots represent the means of eADJ treatment (*n*=3).

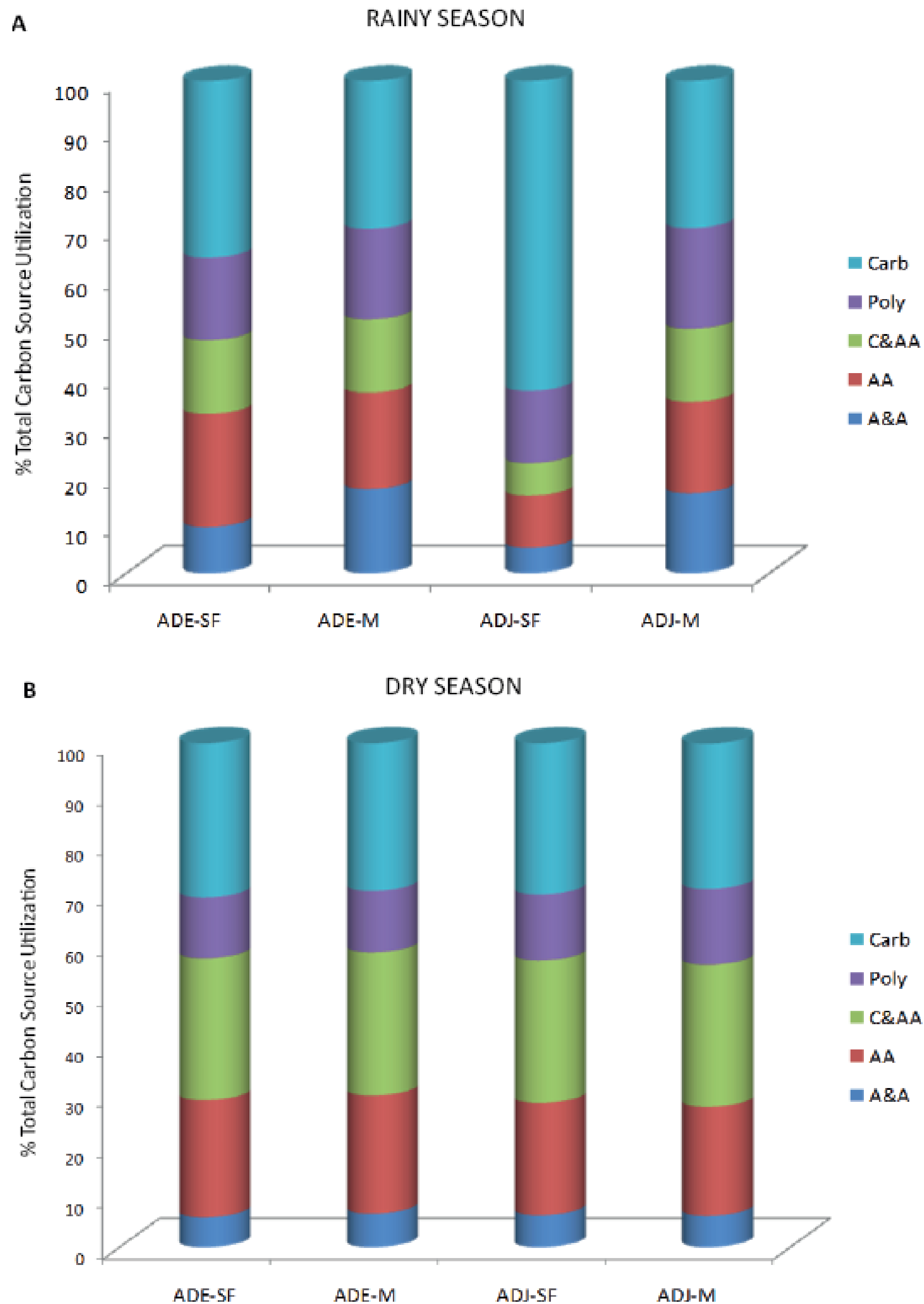

Figure 2. Percent of total carbon source utilization in ADE and ADJ soil samples collected in the rainy season (January 2009) and dry season (August 2009) under secondary forest (SF) and manioc plantation (M) for the different carbon substrate groups: carbohydrates (Carb), polymers (Poly), carboxylic and acetic acids (C & AA), amino acids (AA) and amines and amides (A & A).

ADE samples were not influenced by vegetation type, confirming findings that SOM in ADE is highly stable, even under agricultural use (Woods and McCann, 1999). The large amounts of biochar found in ADE soils are thought to improve and maintain soil fertility by stabilizing organic C in soil and increasing soil C sequestration (Zavalloni et al., 2011).

Soil MBC was significantly higher in ADE. Surprisingly,

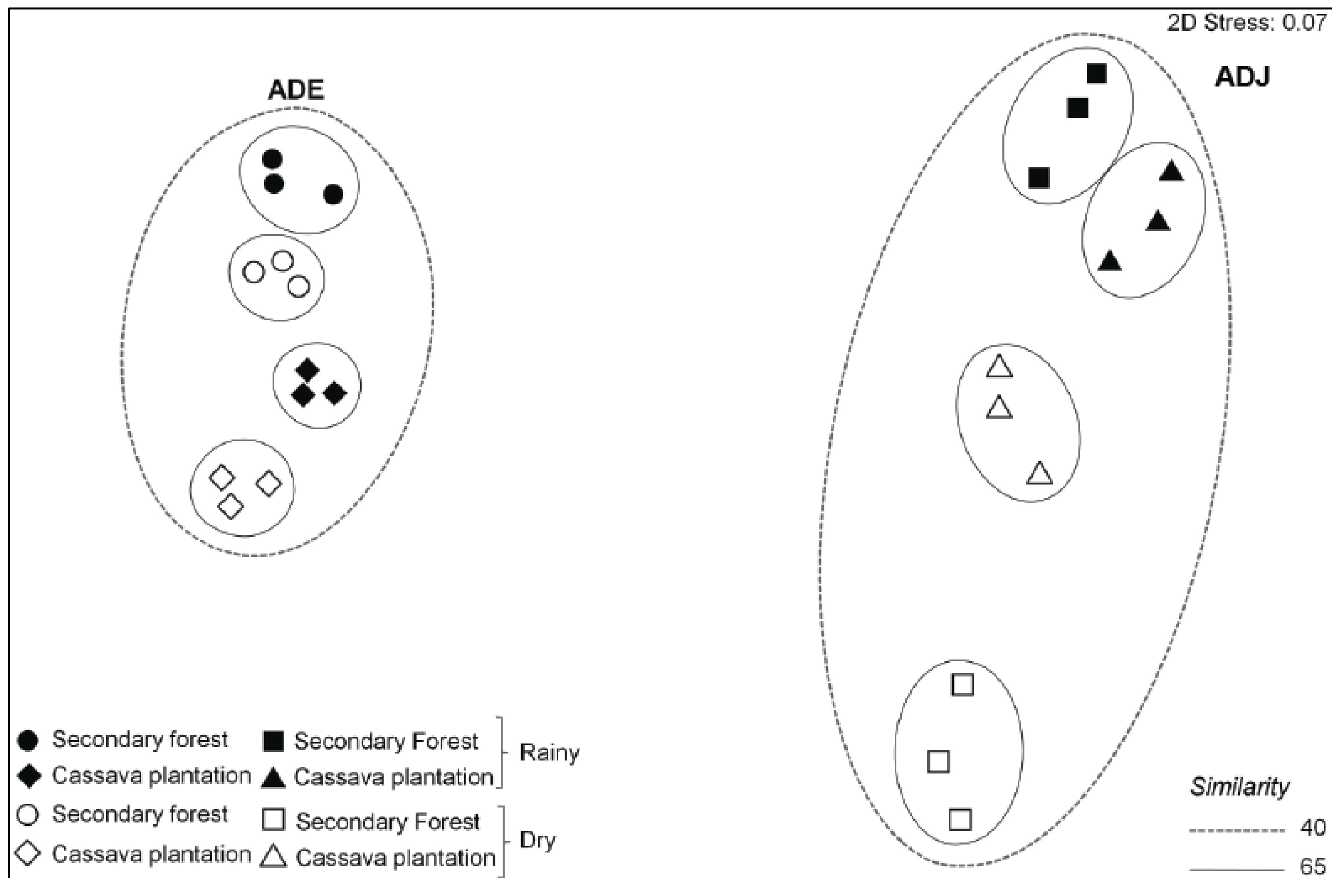

Figure 3. Multidimensional scaling (MDS) ordination based on Bray Curtis similarity analysis of T-RFLP data (square root transformed) of bacterial communities from Amazonian Dark Earth (ADE) and adjacent soil (ADJ) under secondary forest and manioc plantation during the rainy and dry seasons.

Table 2. Percentage of detected bacterial family greater than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial family	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary forest (SF)	Manioc plantation (M)	Secondary forest (SF)	Manioc plantation (M)
Acidobacteria				
Acidobacteriaceae	0.1	0.1	5.0	6.1
Solibacteraceae	1.1	2.1	5.0	5.5
Actinobacteria				
Conexibacteraceae	-	0.2	1.1	1.4
Gaiellaceae	3.6	2.5	0.3	0.3
Micrococcaceae	0.1	-	0.3	2.6
Alphaproteobacteria				
Acetobacteraceae	0.5	0.4	0.7	1.0
Bradyrhizobiaceae	4.4	4.7	7.8	5.5
Caulobacteraceae	0.5	0.5	3.7	1.9
Hyphomicrobiaceae	14.9	10.9	15.7	8.5
Betaproteobacteria				
Burkholderiaceae	0.7	-	5.0	2.1
Oxalobacteraceae	-	0.2	-	3.7
Chloroflexi				

Table 2. Contd

Thermogemmatisporaceae	0.3	1.8	1.9	4.2
Deltaproteobacteria				
Syntrophobacteraceae	4.1	7.8	0.5	0.4
Firmicutes				
Alicyclobacillaceae	1.4	1.5	5.0	3.1
Bacillaceae	0.2	0.3	0.6	1.0
Gammaproteobacteria				
Sinobacteraceae	0.5	0.2	3.9	4.6
Xanthomonodaceae	0.4	0.4	3.2	1.5
Planctomycetes				
Gemmataceae	1.8	2.4	0.3	0.9

Table 3. Percentage of detected bacterial genera greater than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial genus	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary forest (SF)	Manioc plantation (M)	Secondary forest (SF)	Manioc plantation (M)
Acidobacteria				
Candidatus solibacter	1.1	2.1	5.0	5.5
Edaphobacter	0.1	-	0.2	1.7
Firmicutes				
Alicyclobacillus	1.4	1.4	1.9	2.1
Bacillus	0.2	0.3	0.6	1.0
Alphaproteobacteria				
Bradyrhizobium	3.8	4.3	7.3	5.4
Pedomicrobium	2.1	1.4	0.1	0.2
Rhodoplanes	12.1	8.9	15.3	8.3
Betaproteobacteria				
Burkholderia	0.7	-	1.6	0.4
Salinispora	-	-	3.4	1.7
Gammaproteobacteria				
Luteibacter	-	-	2.8	0.4

MBC in ADE-M was not significantly different from ADJ-SF. This suggests that the presence of biochar in ADE soils may enhance MBC (Steiner et al., 2008; Liang et al., 2010). However, there was a clear decline in MBC due to the change in vegetation type for both ADE and ADJ during the rainy season and only for ADJ during the dry season. Such declines in MBC occurring according to the vegetation have been shown in tropical soils of the Central Amazon (Luizão et al., 1992). Seasonal variation in MBC was only observed in ADE-SF (Table 1) with higher values during the rainy season. Cleveland et al. (2004) have reported that high MBC in the rainy season may be controlled by precipitation, which transports the leached organic carbon accumulated in the dry season, thus increasing MBC. However, this effect could not be observed in ADJ-SF, indicating that MBC in ADE-SF acts as a sink during the rainy season; this may be due to high amounts of biochar in ADE combined to plant litter and debris accumulation during the dry season. MBN was strongly affected by land use for both soil types. Low MBN at manioc plantation sites is an indication of enhanced N supply to the plant, and mineral nitrogen is likely to be limited to the MBN. Seasonal variations in MBN were observed at the ADE-M and ADJ-SF sites. August showed very low monthly precipitation (~30 mm), which is less than the average of 58 mm for this month (http://www.bdclima.cnpm.embrapa.br/resultados/index.php). Furthermore, January 2009 reported one of the largest rainfall anomalies in Central Amazonia, between 25 and 50% above normal (Marengo, 2010). This could explain the decline in MBN during the rainy season due to elevated soil moisture (Tiemann and Billings, 2011). Interestingly, ADJ-SF showed no indication of N-mineralization during the rainy season. The presence

Table 4. Spearman rank correlation between selected bacterial family and soil properties.

Soil properties	Acido.	Alicy.	Brady.	Burkh.	Caulo.	Gaiella.	Gemma.	Hypho.	Sino.	Soli.	Syntro.	Thermo.
pH	-0.731*	-0.779**	-0.779**	-0.771**	-0.779**	0.7409*	0.755*		-0.779**	-0.779**	0.826**	-0779**
SOC (g kg^{-1})								0.643*				
SOM (g kg^{-1})								0.640*				
P (mg dm^{-3})	-0.835**			-0.747**		0.765**	0.826**		-0.898***	-0.706*	0.934***	-0.707*
Ca ($cmol_c$ dm^{-3})	-0.764**	-0.640*	-0.643*		-0.635*	0.934***	0.691*		-0.738**	-0.833**		
Mg ($cmol_c$ dm^{-3})	-0.763**	-0.643*	-0.642*		-0.633*	0.934***	0.690*		-0.730**	-0.830**		
Al (g kg^{-1})		0.913*	0.901**	0.919**	0.924**	-0.804**	-0.913***		0.710*	0.710*	-0.736**	0.710*
CEC (g kg^{-1})	-0.763**	-0.643*	-0.632*		-0.633*	0.934***			-0.735**	-0.833**	0.858**	
MBC (g kg^{-1})	-0.812**		-0.634*		-0.630*					-0.929***		-0.929***
MBN (g kg^{-1})	-0.760**					0.682*				-0.881**		-0.881**

[a] *P < 0.05, **P < 0.01, ***P < 0.001. [b] SOC, soil organic carbon; SOM, soil organic matter; CEC, cation exchange capacity; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Acido., Acidobacteraceae; Alicy., Alicyclobacillaceae; Brady., Bradyrhizobiaceae; Burk., Burkholderiaceae; Caulo., Caulobacteraceae; Gaiella., Gaiellaceae; Gemma., Gemmataceae; Hypho., Hyphomicrobiaceae; Sino., Sinobacteraceae; Soli., Solibacteraceae; Syntro., Syntrophobacteriaceae; Thermo., Thermogemmatisporaceae.

of biochar in ADE is probably the main cause of N immobilized in the MBN because no significant changes in SOC and SOM were observed between ADE-SF and ADJ-SF. Steiner et al. (2008) have suggested that N immobilization in biochar amended soils is a desirable phenomenon in soils under heavy rainfall conditions. Furthermore, it is more likely that ADE-SF soils have higher availability of organic C compounds and higher rates of microbial activity, which might trigger N immobilization (Barret and Burke, 2000).

Community functioning as revealed by Biolog

The results of soil function (measured by Biolog substrates) indicate that seasonality has an influence on the metabolism of soil heterotrophic microorganisms (Figures 1 and 2). The patterns of bacterial carbon utilization show that vegetation type and seasonality affected more ADJ than ADE, (Figure 1).

High level of soil moisture observed during the rainy season might have affected the ACH microorganisms (Table 1). Dunn et al. (1985) also observed that physiologically active microorganisms were more sensitive to moist soil rather than dried ones. In addition, cycles of drying and rewetting has been shown affecting the respiration rates in soils, as being significant lower than observed in non-stressed soils (Fierer and Schimel, 2002). Interestingly, ADE bacterial carbon utilization was not influenced by either vegetation and time (Figures 1 and 2), which shows an important feature of ADE soils as usually belowground microbial activity, are very sensitive to soil moisture (Li et al., 2005; Feng et al., 2009). It is also remarkable to state that, despite it is known that substrate utilization is dependent on the initial cell density of the soil inoculums, which can therefore bias subsequent analysis of utilized substrate patterns (Garland, 1996), Biolog plates used here were read after no color development had occurred. Therefore observed differences reliably reflect the ability of a subset of the bacterial community to utilize the Biolog substrates.

Effects of vegetation type and seasonality on bacterial community structure

The bacterial community structure varied with seasonality, with differences observed between the rainy and dry seasons for both, soil and vegetation types, indicating community structure in these soils to be affected by both, moisture and temperature variations (Gordon et al., 2008; Bárcenas-Moreno et al., 2009). MDS demonstrated that seasonality and vegetation affected both soils (Figure 2). It seems that the bacterial communities in ADJ were more sensitive to seasonality, suggesting that ADE communities might be more resistant to such temporal stress. Here, resistant is defined as the ability to withstand a perturbation or stress (McNaughton, 1994). Cruz-Martínez et al. (2009) have indicated that soil microbial communities may be more robust to changes in climate than associated

aboveground macroorganisms. Furthermore, land use appeared to have a stronger effect on structuring the bacterial community in ADJ during the dry season. Perhaps the heavy rainfall in January 2009 (Marengo, 2010) imposed severe stress on the structure of the bacterial communities, diminishing the effect of vegetation. The ADE bacterial community structure appeared to be more affected by vegetation type than seasonality. In agreement with these results, studies in Amazonian tropical soils have shown changes in bacterial community structure according to land use (Jesus et al., 2009; Navarrete et al., 2010). Contrary to these results in other anthropic ADE, Grossman et al. (2010) were not able to detect changes in ADE under different vegetation, which may be explained by the sampling strategy of one single soil horizon or the age of the secondary forest studied.

In addition, BEST analysis in ADE showed correlation with soil P together with MBC and MBN (data not shown). Kuramae et al. (2011) found that P was the major predictor shaping microbial communities in a series of neutral pH fields (pH = 7.0-7.5). Furthermore, Habekost et al. (2008) detected distinct seasonal changes in the microbial community structure; these changes were thought to be driven by the availability and quality of organic resources, which are likely to influence microbial biomass. Interestingly, BEST analysis for ADJ also included MBN as one of the properties shaping the structure of these communities, together with Al, which is known to shape bacterial communities in Amazonian soils (Jesus et al., 2009; Navarrete et al., 2010). Such findings are of great importance for soil management practices, as microbial biomass may act as a sink or source of available N to plants (Friedel et al., 2001).

Effects of vegetation cover and soil type on bacterial community composition

As reported in a previous paper (Taketani et al., 2013), soil type have a stronger selective effect on the class composition of bacterial community, which outpaces the effects imposed by the vegetation. In the present study, the analysis at lower taxonomic level (the family or genus) also demonstrated a stronger effect due to soil type. The most abundant sequences at the family level in ADE soil originated from Gaiellaceae, Gemmataceae and Syntrophobacteraceae. For example, Gaillaceae is a novel family within the class Actinobacteria and what is known is that members of this family are strictly aerobic and chemoorganotrophic (Albuquerque et al., 2011). The chemoorganotrophic bacteria are capable of growing on accumulated organic matter from dead cells and trapped debris which could explain their high abundance in ADE soils, especially under SF. Furthermore, ADJ soils showed higher abundance in nine different groups of family comprising the phyla Acidobacteria, Actinobacteria, Firmicutes and Proteobacteria. Of these, Acidobacteriaceae and Acetobacteriaceae are typical bacteria of acidic environments, in accordance with the low pH of most Amazonian soils and with the highest acidobacterial abundances found in environments with the lowest pH (Fierer et al., 2007; Lauber et al., 2008).

In addition, we also accessed the influence of vegetation cover on the bacterial community composition independently of the soil type. It is well known that microbial communities are not only influenced by soil properties but that plant species also shape the structure and composition of these communities (Berg and Smalla, 2009; Buée et al., 2009; Ladygina and Hedlund, 2010). Interestingly, it was possible to observe the imposed effect of vegetation type on bacterial groups independent of the contrasting soil characteristics of ADE and ADJ.

The bacterial composition of some families and genera smaller than 1% were exclusively detected in ADE soils (Tables S1 and S2). Interestingly, some of these bacterial members are known to play an important role in the carbon and nitrogen cycles. Beijerinckiaceae is a family known to harbor methanotrophs (Dedysh et al., 2000) and seemed to prefer their growth on media of pH 5 (Folman et al., 2008), which is within the pH range of ADE soils. Nitrospiraceae (nitrifying bacteria) was also only observed in ADE and it may indicate that anthropogenic biochar stimulated the presence of bacterial members from this family (Chen et al., 2013). Another particular family detected in ADE was Rhodobiaceae (photoheterotrophic *α-Proteobacteria*) that require carbon under anoxic conditions in light. ADE contains high amounts of anthropogenic biochar and is full of pieces (sherds) of unfired pottery that could increase water-holding capacity and create anoxic microenvironments suitable for bacteria able to grow under these conditions.

Various studies have shown that soil properties influenced microbial communities (Lauber et al., 2008; Singh et al., 2009; Kuramae et al., 2012). In this study, we found that the relative abundance of bacterial families was strongly affected by the differences between the soil properties of ADE and ADJ. One of the main drivers of change in the abundance of the selected bacterial families was soil pH, which is well known to affect soil bacterial communities (Lauber et al., 2009; Singh et al., 2009; Nacke et al., 2011). The strong correlation between Gaiellaceae, Gemmataceae and Syntrophobacteriaceae with soil P also appeared to favor an increase in the abundance of these bacterial groups. This strong correlation with soil P has been previously observed in an old growth forest (DeForest and Scott, 2010), as well as in soils under different land use types (Kuramae et al., 2012).

Conclusion

Concluding, we demonstrated that vegetation cover and seasonality influence the bacterial communities of ADE

and their adjacent soil (Haplic Acrisol, ADJ). The microbial community structure differed in both soils and a higher number of T-RFs were observed in ADE. Average Well Color Development (from Biolog substrates) was higher in ADE than ADJ during the rainy season and kept the same pattern of substrate utilization during the dry season. Considering these results, ADE functional microbial activity was less affected by seasonality. The presence of biochar in ADE likely suggests a buffer effect protecting the system against environmental changes. However, this assumption needs to be further tested with other methods and higher number of samples. Bacterial community composition at deepest taxonomic resolution showed that some groups were in higher abundance or only present in ADE. Taken all together, these results show that ADE maintains important bacterial groups and active bacterial communities. These findings provide insights into microbial community composition, structure and functionality in ADE and their ADJ locations, highlighted by the assessment of how temporal changes in the local environmental conditions and land use types underpin changes in community dynamics.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We thank A.S. Nakatani and D.L.C. Mescolotti for the excellent technical support in the Biolog Ecoplate assay. Many thanks also go to J. Quensen for DNA sample preparation for pyrosequencing. Thanks are also given to R.G. Taketani for assistance with pyrosequencing data analysis. The pyrosequencing was performed by the Michigan State University Genomics Technology Support Facility. We thank A.A. Navarrete, A.C.G. Souza, A.K. Silveira, L.W. Mendes, R.S. Macedo, and T.T. Souza for their help with fieldwork. The authors acknowledge the financial support of CNPq and FAPESP. This research was supported by Embrapa Amazônia Ocidental and by FAPEAM - *Fundação de Amparo à Pesquisa do Estado do Amazonas* doctoral scholarship to the first author. The authors declared no conflict of interest.

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Table S1. Percentage of selected bacterial family smaller than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial family	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary Forest (SF)	Manioc plantation (M)	Secondary Forest (SF)	Manioc plantation (M)
Actinobacteria				
Actinospicaceae	-	-	0.4	0.3
Micromonosporaceae	0.1	0.1	-	-
Nocardioidaceae	0.9	0.3	-	0.2
Patulibacteraceae	0.2	0.1	-	-
Solirubrobacteraceae	0.1	0.1	-	-
Streptomycetaceae	0.3	0.2	0.3	0.1
Thermomonosporaceae	-	0.2	-	0.1
Alphaproteobacteria				
Beijerinckiaceae	0.5	0.2	-	-
Methylocystaceae	0.2	0.1	0.7	0.3
Phyllobacteriaceae	0.1	0.1	-	-
Rhodobiaceae	0.9	0.3	-	-
Xanthobacteraceae	0.2	0.2	-	-
Armatimonadetes				
Chthonomonadaceae	-	0.2	0.1	0.3
Bacteroidetes				
Chitinophagaceae	0.5	0.4	0.5	0.2
Flavobacteriaceae	-	0.1	-	0.1
Firmicutes				
Clostridiaceae	0.4	0.3	0.4	0.2
Paenibacillaceae	0.8	0.2	0.8	0.5
Ruminococcaceae	-	-	0.1	0.1
Sporolactobacillaceae	-	-	0.1	0.1
Thermoactinomycetaceae	0.1	0.1	-	-
Turicibacteraceae	-	-	0.1	0.3
Nitrospirae				
Nitrospiraceae	0.1	0.2	-	-
Planctomycetes				
Isosphaeraceae	0.1	0.1	0.7	0.3
Pirellulaceae	0.5	0.7	0.1	-

Table S2. Percentage of selected bacterial genera smaller than 1% for Amazonian Dark Earth and adjacent soil under secondary forest and manioc plantation.

Bacterial genus	Amazonian Dark Earth (ADE)		Acrisol (ADJ)	
	Secondary Forest (SF)	Manioc plantation (M)	Secondary Forest (SF)	Manioc plantation (M)
Alphaprotebacteria				
Balneimonas	0.3	0.2	-	-
Devosia	0.1	-	0.1	-
Hyphomicrobium	0.2	0.2	0.1	-
Labrys	0.2	0.1	-	-
Rhizobium	0.3	-	-	-
Sphingomonas	0.1	-	-	-
Phenylobacterium	0.1	0.4	0.7	0.3
Acidobacteria				
Acidobacterium	-	-	0.1	-

Table S2. Contd.

Actinobacteria				
Microbacterium	0.1	-	-	-
Nocardioides	0.6	0.1	-	-
Sinomonas	-	-	0.3	0.1
Streptomyces	0.1	0.1	-	-
Terracoccus	-	-	-	0.2
Armatimonadetes				
Chthonomonas	-	0.2	0.1	0.3
Deltaproteobacteria				
Syntrophobacter	-	0.1	-	-
Firmicutes				
Brevibacillus	0.1	-	-	-
Lactobacillus	-	-	0.1	-
Paenibacillus	0.6	0.1	0.7	0.3
Pullulanibacillus	-	-	0.1	0.1
Thermosinus	-	-	0.1	-
Gammaproteobacteria				
Acinetobacter	0.2	-	-	-
Aquicella	0.7	0.2	0.6	0.2
Cupriavidus	-	0.1	-	-
Erwinia	0.3	-	-	-
Lysobacter	0.2	-	-	-
Rhodanobacter	-	-	-	0.6
Stenotrophomonas	0.1	-	-	-
Thermomonas	0.1	-	-	-
Nitrospirae				
Nitrospira	0.1	-	-	-
Planctomycetes				
Gemmata	0.1	0.2	-	-
Verrucomicrobia				
Opitutus	0.1	-	-	-
Pedosphaera	-	-	-	0.1

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