THE ROLE OF MICROBIAL COMMUNITIES IN TROPICAL ECOSYSTEMS

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THE ROLE OF MICROBIAL COMMUNITIES IN TROPICAL ECOSYSTEMS

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The Calakmul Biosphere Reserve in Yucatan contains the archaeological site of Calakmul, one of the largest-known Maya sites, and the largest expanse of seasonal tropical forests remaining in Mexico. The reserve is a ecological gradient conecting the Peten (Guatemala) humid forest with the xeric forest to the north. The function of this ecocline has been challenged by ongoing forest disturbance. Understanding the consequences of land use changes of this biological corridor on soil diversity and microbially-mediated processes is a major conservation frontier. Photo credit: S. Pajares

Tropical ecosystems are different in important ways from those of temperate regions. They are a major reservoir of plant and animal biodiversity and play important roles in global climate regulation and biogeochemical cycling. They are also under great threat due to the conversion of tropical ecosystems to other uses. Thus, in the context of global change, it is crucial to understand how environmental factors, biogeographic patterns, and land use changes interact to influence the structure and function of microbial communities in these ecosystems.

The contributions to this Research Topic showcase the current knowledge regarding microbial ecology in tropical ecosystems, identify many challenges and questions that remain to be addressed and open up new horizons in our understanding of the environmental and anthropological factors controlling microbial communities in these important ecosystems.

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Editorial: The Role of Microbial Communities in Tropical Ecosystems

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The Editorial on the Research Topic

The Role of Microbial Communities in Tropical Ecosystems

Microorganisms represent the largest proportion of the Earth's biodiversity and play an essential role in ecosystem processes, providing functions that ultimately sustain all of life (Falkowski et al., 2008; Prosser, 2012). Understanding the link between ecosystem functioning and the distribution of microbial diversity is essential to predict ecosystem responses to a changing environment (de Vries and Shade, 2013; Logue et al., 2015). With the rapid development of molecular-based techniques, a new interest in understanding the distribution patterns and functional traits of microbial communities has emerged (Fuhrman, 2009; Strickland et al., 2009; Bradford and Fierer, 2012; Krause et al., 2014). However, most of this research has been focused in temperate regions, and the principal mechanisms controlling microbial community variation within the tropics are poorly known.

Tropical ecosystems are different in important ways from those of temperate regions. They are a major reservoir of plant and animal biodiversity and play important roles in global climate regulation and biogeochemical cycling (Gibson et al., 2011; Townsend et al., 2011). They are also under great threat due to the conversion of tropical ecosystems to other uses (Bawa et al., 2004; Stork et al., 2009). Thus, in the context of global change, it is crucial to understand how environmental factors, biogeographic patterns, and land use changes interact to influence the structure and function of microbial communities in these ecosystems.

It is possible that different rules apply to microbial life in tropical ecosystems. For instance, elevated nitrogen deposition by anthropogenic activities may exacerbate phosphorus deficiency in tropical regions, in ways uncommon in temperate ecosystems (Vitousek et al., 2010). However, it is poorly understood how phosphorus availability affects soil microbes (Heuck et al., 2015), or how microbial processes interact with nitrogen deposition in tropical ecosystems (Hietz et al., 2011). Moreover, the distribution of microbes not only is related to environmental factors, but also can vary in relation to temporal and spatial scale (Martiny et al., 2006; Shade et al., 2012). These factors influence biodiversity patterns of larger organisms, but their role in microbial diversity remains unclear (Barberán et al., 2014), especially in tropical systems. In addition, little is known about microbial community responses to disturbance in the tropics. Land use is one of the main drivers of biodiversity alteration in plant and animal communities, especially in tropical areas, where natural ecosystems such as forests are being rapidly altered by conversion to agriculture and other uses (Barnes et al., 2014). Understanding the effects of land use change on soil microorganisms is also a major conservation frontier.

The primary aim of this Research Topic is to explore the environmental and anthropological factors controlling the composition and functional diversity of microbial communities in tropical

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systems. For example, Fanin et al. reported that temporal differences in nitrogen and phosphorus availability in tropical forest soils are critical for the activity and the structure of microbial decomposer communities. Tripathi et al. observed that within tropical rainforests there is a strong degree of ecological differentiation in soil and, as a consequence soil properties together with bacterial and fungal communities varied significantly between forest types. Lee et al. documented the distribution of methanotrophs and methanogens along a depth gradient in a tropical rice paddy, and looked for patterns with oxygen, methane, and organic carbon concentrations in these soils. The influence of environmental and anthropogenic factors, such as nitrogen deposition and land-use change, on the key players in the nitrogen cycle was also assessed in a general review Pajares and Bohannan. This review highlights the large gaps in our understanding of microbially mediated nitrogen processes in tropical forest soils and identifies important areas for future research.

Due to ongoing and widespread deforestation of tropical ecosystems for pasture and crop cultivation, there is an urgent need to evaluate their soil biological diversity. This Research Topic presents new findings on the impact of rainforest conversion on soil microbial community structure, diversity and ultimately functional traits Schneider et al. Particularly in the wet season, land use change from rainforest to agriculture reduced the abundance of different functional microbial groups related to the soil carbon and nitrogen cycles Lammel et al. There is limited knowledge regarding the diversity of important groups of microorganisms in the tropics. Thus, other contributions studied how abundant lineages in soils, such as Acidobacteria Navarrete et al., and Verrucomicrobia Ranjan et al., responded to forest-to-pasture conversion. These studies demonstrate that such groups can be useful as indicators of agricultural impact on tropical ecosystems. Furthermore, the isolation and characterization of two novel moderately thermophilic and acid-tolerant obligate methanotrophs recovered from a tropical methane seep topsoil habitat were described Islam et al.

Some contributions to this Research Topic have focused on how land management practices impact microbially-mediated processes in tropical soils through the alteration of microbial communities. Reverchon et al. showed the influence of different tree plantation systems on nitrogen retention and the abundance of nitrogen functional genes, while Wood et al. concluded that the direct effect of farm management (rather than the indirect effect through changes in the taxonomic and functional diversity of microbial communities) was the dominant control of nutrient loss from smallholder tropical agriculture. Both studies emphasize the necessity of further research on incorporating microbial dynamics into plantation management to improve productivity while mitigating soil fertility loss. Xiong et al. observed that sustainable agricultural management regimes, such as crop rotation, of commercially important crops in tropical regions significantly increased soil fungal diversity and might be a meaningful strategy to prevent vanilla *Fusarium* wilt disease.

Finally, one of the biggest threats facing frog populations in montane tropical systems is a lethal skin disease caused by a chytrid fungus. The composition of the frog skin microbiota can strongly influence many facets of frog health and disease resistance. Field survey data published as part of our Research Topic has demonstrated that the frog skin microbiota is producing broadly similar sets of anti-fungal metabolites across different host species and sites Belden et al.

In summary, the contributions to this Research Topic showcase the current knowledge regarding microbial ecology in tropical ecosystems, identify many challenges and questions that remain to be addressed and open up new horizons in our understanding of the environmental and anthropological factors controlling microbial communities in these important ecosystems.

AUTHOR CONTRIBUTIONS

SP produced the first draft of the editorial, and all authors edited the editorial.

REFERENCES

Barberán, A., Casamayor, E. O., and Fierer, N. (2014). The microbial contribution to macroecology. *Front. Microbiol.* 5:203. doi: 10.3389/fmicb.2014.00203

Barnes, A. D., Jochum, M., Mumme, S., Haneda, N. F., Farajallah, A., Widarto, T. H., et al. (2014). Consequences of tropical land use for multitrophic biodiversity and ecosystem functioning. *Nat. Commun.* 5:5351. doi: 10.1038/ncomms6351

Bawa, K. S., Kress, W. J., Nadkarni, N. M., Lele, S., Raven, P. H., Janzen, D. H., et al. (2004). Tropical ecosystems into the 21st century. *Science* 306, 227–228. doi: 10.1126/science.306.5694.227b

Bradford, M., and Fierer, N. (2012). "The biogeography of microbial communities and ecosystem processes: implications for soil and ecosystem models," in *Soil Ecology and Ecosystem Services*, eds D. H. Wall, R. D. Bardgett, V. Behan-Pelletier, J. E. Herrick, H. Jones, K. Ritz, J. Six, D. R. Strong, and W. H. van der Putten (Oxford: Oxford University Press), 189–200.

de Vries, F. T., and Shade, A. (2013). Controls on soil microbial community stability under climate change. *Front. Microbiol.* 4:265. doi: 10.3389/fmicb.2013.00265

Falkowski, P. G., Fenchel, T., and Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* 320, 1034–1039. doi: 10.1126/science.1153213

Fuhrman, J. A. (2009). Microbial community structure and its functional implications. *Nature* 459, 193–199. doi: 10.1038/nature08058

Gibson, L., Lee, T. M., Koh, L. P., Brook, B. W., Gardner, T. A., Barlow, J., et al. (2011). Primary forests are irreplaceable for sustaining tropical biodiversity. *Nature* 478, 378–381. doi: 10.1038/nature10425

Heuck, C., Weig, A., and Spohn, M. (2015). Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus. *Soil Biol. Biochem.* 85, 119–129. doi: 10.1016/j.soilbio.2015.02.029

Hietz, P., Turner, B. L., Wanek, W., Richter, A., Nock, C. A., and Wright, S. J. (2011). Long-term change in the nitrogen cycle of tropical forests. *Science* 334, 664–666. doi: 10.1126/science.1211979

Krause, S., Le Roux, X., Niklaus, P. A., van Bodegom, P. M., Lennon, T., J. T., Bertilsson, S., et al. (2014). Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. *Front. Microbiol.* 5:251. doi: 10.3389/fmicb.2014.00251

Logue, J. B., Findlay, S. E. G., and Comte, J. (2015). Editorial: microbial responses to environmental changes. *Front. Microbiol.* 6:1364. doi: 10.3389/fmicb.2015.01364

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., et al. (2006). Microbial biogeography: putting microorganisms

on the map. *Nat. Rev. Microbiol.* 4, 102–112. doi: 10.1038/nrmicro1341

Prosser, J. I. (2012). Ecosystem processes and interactions in a morass of diversity. *FEMS Microbiol. Ecol.* 81, 507–519. doi: 10.1111/j.1574-6941.2012.01435.x

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Bürgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3:417. doi: 10.3389/fmicb.2012.00417

Stork, N. E., Coddington, J. A., Colwell, R. K., Chazdon, R. L., Dick, C. W., Peres, C. A., et al. (2009). Vulnerability and resilience of tropical forest species to land-use change. *Conserv. Biol.* 23, 1438–1447. doi: 10.1111/j.1523-1739.2009.01335.x

Strickland, M. S., Lauber, C., Fierer, N., and Bradford, M. A. (2009). Testing the functional significance of microbial community composition. *Ecology* 90, 441–451. doi: 10.1890/08-0296.1

Townsend, A. R., Cleveland, C. C., Houlton, B. Z., Alden, C. B., and White, J. W. C. (2011). Multi-element regulation of the tropical forest carbon cycle. *Front. Ecol. Environ.* 9, 9–17. doi: 10.1890/100047

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. (2010). Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecol. Appl.* 20, 5–15. doi: 10.1890/08-0127.1

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(A)synchronous Availabilities of N and P Regulate the Activity and Structure of the Microbial Decomposer Community

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Nitrogen (N) and phosphorus (P) availability both control microbial decomposers and litter decomposition. However, these two key nutrients show distinct release patterns from decomposing litter and are unlikely available at the same time in most ecosystems. Little is known about how temporal differences in N and P availability affect decomposers and litter decomposition, which may be particularly critical for tropical rainforests growing on old and nutrient-impoverished soils. Here we used three chemically contrasted leaf litter substrates and cellulose paper as a widely accessible substrate containing no nutrients to test the effects of temporal differences in N and P availability in a microcosm experiment under fully controlled conditions. We measured substrate mass loss, microbial activity (by substrate induced respiration, SIR) as well as microbial community structure (using phospholipid fatty acids, PLFAs) in the litter and the underlying soil throughout the initial stages of decomposition. We generally found a stronger stimulation of substrate mass loss and microbial respiration, especially for cellulose, with simultaneous NP addition compared to a temporally separated N and P addition. However, litter types with a relatively high N to P availability responded more to initial P than N addition and *vice versa*. A third litter species showed no response to fertilization regardless of the sequence of addition, likely due to strong C limitation. Microbial community structure in the litter was strongly influenced by the fertilization sequence. In particular, the fungi to bacteria ratio increased following N addition alone, a shift that was reversed with complementary P addition. Opposite to the litter layer microorganisms, the soil microbial community structure was more strongly influenced by the identity of the decomposing substrate than by fertilization treatments, reinforcing the idea that C availability can strongly constrain decomposer communities. Collectively, our data support the idea that temporal differences in N and P availability are critical for the activity and the structure of microbial decomposer communities. The interplay of N, P, and substrate-specific C availability will strongly determine how nutrient pulses in the environment will affect microbial heterotrophs and the processes they drive.

Keywords: decomposition, microbial limitations, multiple element limitation, nutritional constraints, nutrient availability, successive fertilization, temporal variability

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INTRODUCTION

Nutrient availability often exerts strong limitations on ecological processes in different ecosystem types and across distinct biomes (Vitousek and Howarth, 1991; Elser et al., 2007). Nitrogen (N) and phosphorus (P) play a particularly important role because of their high requirements for building living biomass, and because they determine growth processes and biological activity to a large degree (Sterner and Elser, 2002; Hartman and Richardson, 2013). An important source of N and P, especially for P with ongoing ecosystem and soil development (Walker and Syers, 1976), is dead organic matter of mostly plant origin. Due to the large variability in leaf C: N: P stoichiometry from high to low latitudes (McGroddy et al., 2004), and among phylogenetic groups, life forms or plant species (Hättenschwiler et al., 2008; Yuan and Chen, 2009), microbial decomposers are exposed to substrates of a considerable range in C: N: P (Cleveland and Liptzin, 2007). Recently, Fanin et al. (2013) proposed that C: N: P of leaf litter leachates also control the stoichiometry and structure of the microbial decomposer community. This finding highlights the importance of considering the relevant substrate used by microbial decomposers during the initial stage of decomposition, with leachate C: N: P stoichiometry varying from 169:2:1 to 8280:96:1 among different litter species in the studied tropical rainforest of French Guiana (Fanin et al., 2014). Thus, besides the commonly measured stoichiometry of bulk leaf litter material, the variability of N and P in soluble compounds is likely critical for assessing the effects of any alterations in nutrient availability on microbial communities.

Simultaneous additions of N and P in tropical forests were shown to strongly stimulate litter mass loss and microbial functioning during decomposition (Hobbie and Vitousek, 2000; Reed et al., 2011; Barantal et al., 2012; Fanin et al., 2012). These results support the increasing evidence that ecosystem processes are often co-limited by N and P rather than by N or P alone (Vitousek et al., 2010; Sundqvist et al., 2014). In contrast to the simultaneous application of N and P during fertilization experiments, these two nutrients are unlikely to be available in balanced amounts at the same time in most ecosystems due to their distinct quantities and chemical bonds in dead organic matter and because of their different pathways during biogeochemical cycling. For example, in contrast to N that forms relatively strong chemical bonds with C atoms, ester bonds between P and C atoms are comparatively easier to break, allowing microorganisms to mineralize P from organic molecules with less energy than is required to mineralize N (McGill and Cole, 1981). Thus, although P availability is relatively in excess initially, it may become quickly limiting during the decomposition process. Nutrient availability may also vary throughout the year, with local leaf litter input changing significantly in N: P stoichiometry and in the amount of dissolved organic C between seasons (Wood et al., 2005; Cleveland and Townsend, 2006; Cleveland et al., 2006). Similarly, N or P deposition originating from fossil fuel use, agricultural activity, or from Saharan dust are projected to rise in tropical biomes (Galloway et al., 1994; Okin et al., 2004), but these nutrient inputs are likely to vary substantially over time. For example, P deposition is strongly dependent on Saharan climate and global circulation patterns and has been shown to vary by almost twofold from 20 to 35 kg ha^{-1} $year^{-1}$ (Okin et al., 2004). Despite the large temporal variability in the relative availability of C and nutrients over quite short time spans, its consequences for the structure and function of soil microbial communities remain poorly studied.

Because plant leaf litter represents the most important source of C and nutrients for microbial decomposers, any modification of substrate stoichiometry and nutrient availability can influence extracellular enzyme production (Allison and Vitousek, 2005; Mooshammer et al., 2012; Sinsabaugh et al., 2015), and element-use efficiency of decomposer communities (Manzoni et al., 2012; Mooshammer et al., 2014a). Recently, empirical and theoretical studies showed that shifts in the microbial community structure and in the stoichiometry of the microbial biomass could be one way microbes respond to stoichiometric imbalance with their substrates (Fanin et al., 2013; Kaiser et al., 2014). Because bacteria and fungi present different stoichiometric requirements and constraints (Keiblinger et al., 2010; Strickland and Rousk, 2010), any changes in N and P availability may strongly control the relative proportion of these groups within the community (Krashevska et al., 2010). For instance, Güsewell and Gessner (2009) showed in a microcosm experiment that bacteria were relatively more abundant when decomposition of cellulose was N limited, whereas the relative abundance of fungi increased when cellulose decomposition was P limited. However, whether the timing of N and P availability is critical for the structure and functioning of microbial communities have received only scant attention.

Here we examined how microbial communities respond to successive N and P additions, and whether these responses differ among leaf litter substrates with distinct leachate C: N: P stoichiometry and cellulose as a nutrient free substrate. We specifically addressed the following question: Do successive N and P additions (first N and then P, and *vice-versa*) have different effects compared to simultaneous N and P additions? Although we anticipated that a combined NP addition would stimulate microbial activity more than a temporally separated N and P addition, we hypothesized that this stimulation would be dependent on the substrate quality. Specifically, we anticipated that the effects of sequential nutrient supply would depend on the N: P ratio of the soluble fraction of the litter substrates: (i) litter that is relatively rich in soluble N or P would respond stronger when P or N is added first, respectively, (ii) litter that is relatively well balanced in soluble N and P would not show a particular response to either N or P added first, and (iii) cellulose as a nutrient-free substrate would display the strongest effects when both nutrients are added simultaneously (Hypothesis 1). Because these two nutrients show distinct release patterns from decomposing litter, we hypothesized that the order in the relative N- and P-availability would determine microbial process rates, with: (i) P added first and then N resulting in the weakest effect because P is relatively available early during the decomposition process, (ii) N added first and then P showing an intermediate effect as P is relatively more limiting later during the decomposition process, and (iii) a simultaneous NP addition

having the strongest effects because of synergistic responses when both elements are available in excess (Hypothesis 2). Finally, as a result of different nutrient requirements between fast-growing bacteria and more slowly growing fungi (Güsewell and Gessner, 2009; Keiblinger et al., 2010), we hypothesized that the relative abundance of these different microbial groups would be altered in a predictable way by N and P additions, with: (i) P added first and then N leading first to an increased proportion of bacteria that decreases again when N is added (ii) N added first and then P increasing the relative proportion of fungi in a first time which decreases again once P is added, (iii) simultaneous NP addition stimulating both fungal and bacterial biomasses with no net change in their relative abundance (Hypothesis 3). We tested our hypotheses with a laboratory microcosm experiment by measuring litter mass loss, microbial activity (substrate-induced respiration, SIR), and microbial community structure (phospholipid fatty acids, PLFAs) in the litter and the underlying soil during the initial stage of decomposition.

MATERIALS AND METHODS

Soil and Litter Material

The soil used for our experiment was collected from the topsoil (0–10 cm) in the Amazonian rainforest, at Paracou, French Guiana (5°15′N, 52°53′W). It is classified as a nutrient-poor acrisol developed over a Precambrian metamorphic formation called the Bonidoro series with a mean pH of 4.4 in the top soil and a soil texture with 20% clay, 6% silt and 74% sand, with a total C of 22.1 g kg^{-1}, a total N of 1.5 g kg^{-1} soil and a total P of 0.10 g kg^{-1} soil (see Fanin et al., 2011 for more details). The soil was dried at 25°C, sieved at 2 mm, homogenized, and stored dry until use.

Air-dried leaf litter of three different tree species occurring at our research site [*Goupia glabra* (Aublet), *Simarouba amara* (Aublet) and *Vochysia tomentosa* (G. Mey.)] as well as cellulose paper (filter paper qualitative 410, VWR, Fontenay-sous-Bois, France) were used as substrates for decomposition. In addition to leaf litter as a naturally occurring substrate for microbial growth, we used cellulose as a widely accessible C source for a large part of the heterotrophic microbial community that contains no other elements than C, O and H. Cellulose served as an extreme end point along the stoichiometric gradient covered by the three litter species and also to add a single C compound compared to the litter species composed of diverse and distinct C compounds (**Table 1**). The three litter species were selected based on their distinct C: N: P stoichiometry of the soluble fraction (**Table 1**) that has previously been shown to have a major impact on litter and soil microbial communities (Fanin et al., 2013, 2014). The three species also showed contrasting responses to combined N and P fertilization in a previous decomposition experiment in the field, with a very small effect on *V. tomentosa*, an intermediate effect on *S. amara,* and a strong effect on *G. glabra* (Fanin et al., 2012).

Freshly fallen leaf litter was collected with 25 m × 25 m large litter traps installed in mono-specific stands of an experimental plantation adjacent to the Paracou natural forest station (Roy et al., 2005), air-dried immediately upon collection, pooled across sampling dates, and stored dry. Only intact leaves without signs of herbivory, galls or fungal attack were kept (see Fanin et al., 2012 for further details on leaf litter collection).

Experimental Design

For testing the impact of the changing relative availability of N and P through time on decomposition and microbial decomposer communities, we defined three different fertilization treatments. The first treatment consisted in the combined addition of N and P at the same time (denoted as '*NP*' in the following). In the second treatment we applied first N and later P (denoted as '*Np*' in the following), and in the third treatment we applied first P and later N (denoted as '*Pn*' in the following). A control treatment without any fertilizer application was also included. Nutrients were added either at the beginning of the experiment (d_0), and/or 36 days after the start of experiment (d_{36}) depending on the treatment. We added N as KNO_3 and NH_4NO_3, and P as KH_2PO_4 at the same total quantities of N and P as used in a fertilization experiment in the field at the research site in Paracou (Barantal et al., 2012), i.e., 0.055 g KNO_3 and 0.069 g NH_4NO_3 per microcosm, and 0.074 g KH_2PO_4 per microcosm. We used KNO_3 in addition to NH_4NO_3 to keep the added K constant between the N and P treatments. The total amounts of added N and P correspond to 130 kg N ha^{-1} y^{-1}, and 69 kg ha^{-1} y^{-1}, respectively, which is comparable to the quantities used in other fertilization experiments in tropical forests (see Hobbie and Vitousek, 2000; Cleveland et al., 2006; Kaspari et al., 2008).

The microcosms consisted of 30 cm wide × 15 cm long × 10 cm tall plastic boxes that were covered with a plastic cap. Each microcosm received 80 g of homogenized air dry soil and 4 g (oven dried-corrected weight) of air dry leaf litter material or cellulose paper that was placed on the top of the soil. Leaf litter and cellulose paper were cut in 1 cm × 1 cm squares in order to homogenize leaf margin: total surface area ratio across all substrates. For each microcosm, we soaked the 4 g-substrate aliquotes in 40 mL of distilled water (for the *control* treatment) or in 40 mL of the corresponding fertilizer solution (for the *NP*, *Np,* and *Pn* treatments) while shaking for 24 h in order to ensure complete and homogeneous rewetting of the substrates before their addition to the microcosms. Along with the substrates we also added the soaking solution to each individual microcosm at d_0, which corresponded to the volume of water needed to reach 80% of water holding capacity of the soil in the microcosm. For the second fertilization event at d_{36}, 10 mL distilled water or fertilizer solutions (four times more concentrated than at d_0 in order to add the same quantity of nutrients while keeping soil humidity close to 80% of water holding capacity) were added to all microcosms of the respective treatments. During the entire experimental duration of 74 days, the microcosms were kept at constant temperature of 30°C and under water saturated atmosphere, and microcosms were sprinkled to their original weight with distilled water every 4 days in order to keep soil and litter humidity close to constant.

We constructed a total of 12 microcosms for each combination of litter material (*G. glabra, S. amara, V. tomentosa* and cellulose paper) and fertilization treatment (control, *NP*, *Np,* and *Pn*),

resulting in a total of 192 microcosms. Three microcosms for each treatment combination were harvested after 18 (d_{18}) and 36 (d_{36}) days, just before the second fertilization event, and after 54 (d_{54}) and 74 days of incubation (d_{74}).

Response Variables

At each sampling date and for each microcosm, the remaining litter material was carefully retrieved, cleaned from soil particles, and weighed for fresh remaining mass. A litter subsample was dried for 48 h at 65°C for dry mass conversion and the calculation of litter mass loss in each microcosm. Additional subsamples of litter and of homogenized soil were immediately frozen after pooling the three replicates of each treatment for further PLFA analyses ($n = 1$ for each litter × treatment combination). The remaining material was air-dried at 25°C.

Substrate induced respiration (SIR), as an integrative indicator of the overall microbial activity (Nannipieri et al., 2003), was determined for soil and litter material as previously described (Fanin et al., 2012). Briefly, 10 g of air dry soil were placed in a sealed 150 mL flask and received a solution of glucose in order to reach 80% of the soil water holding capacity and to add 1.5 mg C g^{-1} of soil. For litter materials, 2 g of air dry litter was incubated in the same way, but adding glucose solution to reach 20 mg C g^{-1} of litter. The flasks were incubated at 25°C for 6 h, a time span that is considered as short enough to avoid *de novo* enzyme synthesis. Two hundred micro liter headspace air samples were analyzed after 2 and 6 h for CO_2 concentration using a gas chromatograph with catharometric detection (Varian CP-4900, Walnut Creek, CA, USA). The amount of CO_2 released during the 4 h time lapse was used to calculate SIR rate expressed in μg C-CO_2 g^{-1} soil or litter h^{-1}.

Microbial community structure was determined by analyzing group-specific PLFAs from a representative subsample of 1 and 10 g fresh weight frozen litter material and soil, respectively, using the protocol described in Fanin et al. (2014). Branched and saturated, mono-unsaturated and cyclopropyl PLFAs i15:0, a15:0, i16:0, i17:0, a17:0 16:1ω7c, cy17:0, 18:1ω7c, cy19:0 were used to characterize bacteria while the 18:1ω9, 18:2ω6,9 PLFAs were used as biomarkers of fungi (e.g., Frostegård and Bååth, 1996; Zelles, 1999; Bååth, 2003).

Data Analysis

We analyzed litter mass loss, litter SIR, and soil SIR at the different sampling dates using repeated measures ANOVAs in which litter material, fertilizer treatment, and sampling date were treated as fixed effects and were permitted to interact. Microcosm identity was included as a random factor to account for the repeated sampling over time. For each litter material, we also compared the differences between treatments for each sampling date, followed by a *post hoc* test of Tukey-HSD ($\alpha = 0.05$). Using Bray–Curtis dissimilarity matrices on the selected 23 PLFAs markers, we used permutational multivariate ANOVAs (PERMANOVA) to test the effects of treatment, litter material and sampling date on microbial community structure. Following repeated measure ANOVA, we used the % sum of square as a comparative

TABLE 1 | Chemical characteristics of litter species (mean ± SD) used for the microcosm incubations.

Litter traits	Cellulose	Goupia glabra	Simarouba amara	Vochysia tomentosa
Bulk material				
C (mg g^{-1})	445	497 ± 2	491 ± 1	429 ± 4
N (mg g^{-1})	0	12, 1 ± 1, 3	11, 1 ± 0, 7	8, 7 ± 0, 4
P (mg g^{-1})	0	0, 33 ± 0, 04	0, 32 ± 0, 02	0, 29 ± 0, 01
C: N	∞	41, 1 ± 4, 1	44, 2 ± 2, 9	49, 3 ± 2, 9
C: P	∞	1507 ± 168	1534 ± 111	1479 ± 87
N: P	–	36, 7 ± 1, 5	34, 7 ± 0, 7	30 ± 2, 9
C: N: P	–	1507:37:1	1534:35:1	1479:30:1
Soluble fraction				
solC (mg g^{-1})	–	19, 3 ± 2, 5	10, 7 ± 0, 6	7, 5 ± 0, 3
solN (mg g^{-1})	–	54, 2 ± 3, 5	125, 0 ± 8, 3	86, 4 ± 3, 9
solP (mg g^{-1})	–	46,1	36,4	0,9
solC: N	∞	355, 5 ± 25, 8	85, 8 ± 5, 3	86, 3 ± 2, 4
solC: P	∞	419 ± 54	294 ± 15, 9	8280 ± 297
solN: P	–	1, 2 ± 0, 1	3, 4 ± 0, 2	96, 0 ± 4, 4
solC: N: P	–	419:1:1	294:3:1	8280:96:1
Carbon forms				
Water soluble compounds (%DM)	–	36.6 ± 0.4	45.4 ± 0.4	34.6 ± 1.1
Hemicellulose (%DM)	–	16.2 ± 0.7	11.7 ± 0.2	20.1 ± 1.1
Cellulose (%DM)	100	18.8 ± 0.3	20.0 ± 0.3	19.7 ± 0.4
Lignin (%DM)	–	28.4 ± 0.8	22.8 ± 0.7	25.6 ± 0.4
Total phenolic (%DM)	–	1.1 ± 0.2	4.4 ± 0.2	0.6 ± 0.1
Soluble phenolic (%DM)	–	2.8 ± 0.3	11.0 ± 0.8	4.4 ± 0.4
Tannin (%DM)	–	0.6 ± 0.1	6.3 ± 0.3	3.9 ± 0.3

measure of % variation explained by the different factors (substrate identity, fertilization treatment, sampling date and their interactions). We run non-metric multidimensional scaling (NMDS) with vector fitting, based on multiple linear regressions using the coordinates on the first two principal axes as the explanatory variables, and the variable of interest (e.g., relative abundance of microbial groups) as the dependent variable. Finally, to visualize the effect of N and P availability on microbial community structure, we compared the fungi to bacteria ratio (fungi:bacteria) across all substrate types as a function of the different fertilization treatments at d_{36} and d_{74}. Cellulose was excluded from this analysis because of the large differences in microbial community responses compared to the three other species. All statistical tests were performed with the R software (version 2.11.1).

RESULTS

Fertilization and Substrate Identity Effects on Litter Mass Loss

Sampling date (28.4% of the variation explained), fertilization treatment (16.1%), and substrate identity (6.1%) all had significant effects on substrate mass loss (**Table 2A**). The interaction between substrate identity and fertilization treatment also explained 11.3% of the observed variation in substrate mass loss, i.e., a higher amount of variation than the substrate identity alone. Of all substrates, cellulose showed the largest variation in mass loss among the fertilization treatments, ranging from 22.5% of initial mass lost on average in the control treatment to 57.4% of initial mass lost in the *NP* treatment at the final harvest after 74 days of decomposition (**Figure 1A**). The other two fertilization treatments where N and P were added successively showed intermediate final mass loss of 32.6% for *Pn* and 40.6% for *Np*. Despite the marginally significant interaction between sampling date and fertilization treatment (**Table 2A**), cellulose mass loss in response to N or P addition (as the first phase of the *Np* or *Pn* treatments, respectively) did not differ from the control treatment during the first 36 days (**Figure 1A**). After the additional fertilization event (i.e., when the second nutrient was added), cellulose mass loss was faster in both treatments (*Np* and *Pn*) compared to the control treatment, although still remaining lower as compared to cellulose fertilized with both nutrients simultaneously. Opposite to cellulose, *V. tomentosa* leaf litter was not affected by any of the fertilization treatments (**Figure 1D**). *G. glabra* and *S. amara* leaf litters showed intermediate responses to the fertilization treatments compared to cellulose and *V. tomentosa* leaf litter. After 74 days, mass loss in the *NP* treatment was 13.7 and 19.4% higher than in the control for *G. glabra* and *S. amara*, respectively (**Figures 1B,C**). Adding P first clearly enhanced decomposition of *S. amara* but not that of *G. glabra* during the first part of the experiment. In both, *Np* and *Pn* treatments, the addition of the second nutrient at d_{36} influenced further decomposition only little, and the differences to mass loss in the control treatment got overall rather smaller with ongoing experimental duration.

Fertilization and Substrate Identity Effects on SIR

Substrate induced respiration in decomposing material varied between 1.8 and 258.6 $\mu g\ C\text{-}CO_2\ g^{-1}$ across all substrates and all sampling dates (**Figure 2**). In contrast to litter mass loss, litter SIR was primarily influenced by substrate identity and its interaction with sampling date, explaining 31.8 and 10.8% of the overall variation, respectively (**Table 2B**). A significant, though lower amount of variation in litter SIR was explained by fertilization treatment (10.6%) and its interaction with sampling date (8.5%). Prior to the second fertilization event, SIR rates were highest in cellulose in the NP treatment (49.2 $\mu g\ C\text{-}CO_2\ g^{-1}$ at d_{36}), while SIR rates following the supply of N or P alone did not differ from the control treatment between 0 and 36 days (**Figure 2A**). When the second nutrient was added, SIR increased to 94.0 and 66.8 $\mu g\ C\text{-}CO_2\ g^{-1}$ at d_{54} in the *Pn* and *Np* treatments, respectively, reaching values similar to those when cellulose was fertilized with N and P at the same time (*NP* treatment). Without any nutrient addition (control treatment), SIR rates on cellulose were null at all of the sampling dates. Similar to cellulose, *G. glabra* showed a substantial stimulation of litter SIR following the first fertilization event when N and P were added together, reaching 223.9 $\mu g\ C\text{-}CO_2\ g^{-1}$ at d_{36} on average (**Figure 2B**). After the second fertilization, SIR rates of *G. glabra* in *Pn* and *Np* treatments were higher than in the control and did not differ to those observed in *NP*. But on d_{74} the stimulation of SIR was highest in *NP* and intermediate in *Np* and *Pn* as compared to the control. Similar to cellulose and *G. glabra*, SIR rates of *V. tomentosa* and *S. amara* leaf litter were also higher at d_{36} with a combined *NP* fertilization than with N supplied alone, and also with higher SIR rate when P was added compared to N (**Figures 2C,D**). Following the second fertilization, and as observed for cellulose and *G. glabra*, SIR rates at d54 increased following P supply in the *Np* treatments for both litter species, and with N supply in the *Pn* treatment on *S. amara*, and reached similar values to those observed when both resources were added simultaneously (*NP* treatment). But at the final d_{74} sampling, the stimulation of SIR rates of *V. tomentosa* following *Np* did not persist.

Substrate induced respiration measured in the soil underneath the decomposing substrates was generally distinctively lower than that measured in decomposing material, varying between 0.70 and 6.59 $\mu g\ C\text{-}CO_2\ g^{-1}$ soil (**Figure 3**). The hierarchy of factors explaining soil SIR over the incubation period were similar to those observed for litter SIR, with a large part of the variation explained by substrate identity (31.3%), fertilization treatment (11.3%) and sampling date (5.6%) as well as interactions between factors (**Table 2C**). Overall, *NP* addition and *P* only addition treatments showed higher soil SIR rates at d_{36} compared to N only addition or control treatment, except for cellulose where *NP* did not differ from the control (**Figure 3A**). The addition of N in the *Pn* treatment did not show any influence on the soil SIR rates under *V. tomentosa*, *G. glabra* and cellulose, and even a trend for a negative effect under *S. amara* (**Figure 3C**). Opposite to this pattern, the addition of P in the *Np* treatment stimulated soil SIR rates, especially under *G. glabra* with an increase from 2.40 to 5.28 $\mu g\ C\text{-}CO_2\ g^{-1}$ between d_{36} and d_{54} (**Figure 3B**). Differences between treatments were, however, smaller at the end

TABLE 2 | Repeated measures ANOVA evaluating the role of substrate identity, treatment and sampling dates: (A) Litter mass loss, (B) SIR Litter, (C) SIR Soil.

	df	%SS	SS	MS	F.model	*p-value*
(A) Litter mass loss						
Substrate identity	3	6.1	1627	542.3	7.6	<0.0001
Fertilization treatment	3	16.1	4324	1441.4	20.2	<0.0001
Sampling date	3	28.4	7625	2541.6	35.6	<0.0001
Substrate identity × Fertilization treatment	9	11.3	3038	337.6	4.7	<0.0001
Fertilization treatment × Sampling date	9	4.3	1146	127.3	1.8	0.078
Substrate identity × Sampling date	9	1.5	405	44.9	0.6	0.77
Residuals	122	32.4	8710	71.4		
(B) SIR Litter						
Substrate identity	3	31.8	9.5	3.2	56.9	<0.0001
Fertilization treatment	3	10.6	3.2	1.1	18.9	<0.0001
Sampling date	3	5.9	1.8	0.6	10.5	< 0.0001
Substrate identity × Fertilization treatment	9	5.2	1.6	0.2	3.1	0.002
Fertilization treatment × Sampling date	9	8.5	2.5	0.3	5.1	< 0.0001
Substrate identity × Sampling date	9	10.8	3.2	0.4	6.5	< 0.0001
Residuals	146	27.2	8.1	0.1		
(C) SIR Litter						
Substrate identity	3	31.3	107.8	35.9	59.4	<0.0001
Fertilization treatment	3	11.3	39.0	13.0	21.5	<0.0001
Sampling date	3	5.6	19.3	6.4	10.6	<0.0001
Substrate identity × Fertilization treatment	9	7.6	26.4	2.9	4.8	<0.0001
Fertilization treatment × Sampling date	9	7.6	26.2	2.9	4.8	<0.0001
Substrate identity × Sampling date	9	11.0	37.9	4.2	7.0	<0.0001
Residuals	146	25.6	88.4	0.6		

Repeated measures ANOVAs were performed to examine the relative influence of litter material, fertilization treatment, and time, as well as interactions between the three explanatory variables in accounting for the variation in microbial activity over the course of the incubation experiment.

of the incubation, except for *V. tomentosa* with a higher soil SIR rate of 5.19 in *NP* compared to 2.05 in the control (**Figure 3D**).

Fertilization and Substrate Identity Effects on the Microbial Community Structure

The structure of the microbial community (i.e., relative abundance of the 23 group-specific PLFA markers) differed strongly among the decomposing substrates, with contrasted NMDS plots mostly driven by cellulose that showed a distinct PLFA pattern compared to leaf litter along the second NMDS axis (**Figure 4A**). Such differences in microbial community structure between leaf litter and cellulose were clearly related to lower fungal lipid markers in leaf litter than in cellulose that translated into lower litter fungi:bacteria (see Supplementary Table S1 for more details). PERMANOVA to test for the effects of the different treatments on PLFA markers revealed that substrate identity accounted for 42.1% of the overall variation of the microbial community structure, which is roughly as much as that accounted for by all the remaining factors and interactions (**Table 3A**). To a lower extent, the structure of the microbial community in the decomposing substrates also differed across the fertilization treatments and their interactions with sampling date and substrate identity (6.4, 20.1, and 13.8% of explained variation, respectively). When omitting the cellulose substrate from the comparison, the effects of fertilization treatments were particularly apparent on the leaf litter fungi:bacteria (**Figure 4B**). The addition of N only strongly decreased the relative proportion of bacterial lipid markers by 49% whereas the addition of P only increased it by 57% on average. Such change resulted in contrasting fungi:bacteria of 7.1 (with N only) or 1.7 (with P only), respectively, compared to 2.8 in the control treatment. After complementary P addition in the *Np* treatment or N addition in the *Pn* treatment, the fungi:bacteria shifted to almost similar values. No significant difference was observed among the fertilization treatments at d_{74}.

Microbial community structure in the soil was affected by different factors compared to that in decomposing substrates (**Table 3B**). Neither substrate identity nor fertilization treatment and their interactions explained the PLFA-based microbial community structure in the soil. In contrast, sampling date and its interaction with fertilization treatment accounted for 18.7 and 10.7% of the total variation observed in soil microbial community structure, respectively. We did not find any clear effect of the substrate identity on the soil community structure in the NMDS plot (**Figure 5A**). By contrast to the litter microbial community and even after omitting the cellulose substrate, we did not find any clear effects of fertilization or sampling date on the PLFA-based soil microbial community structure (**Figure 5B**).

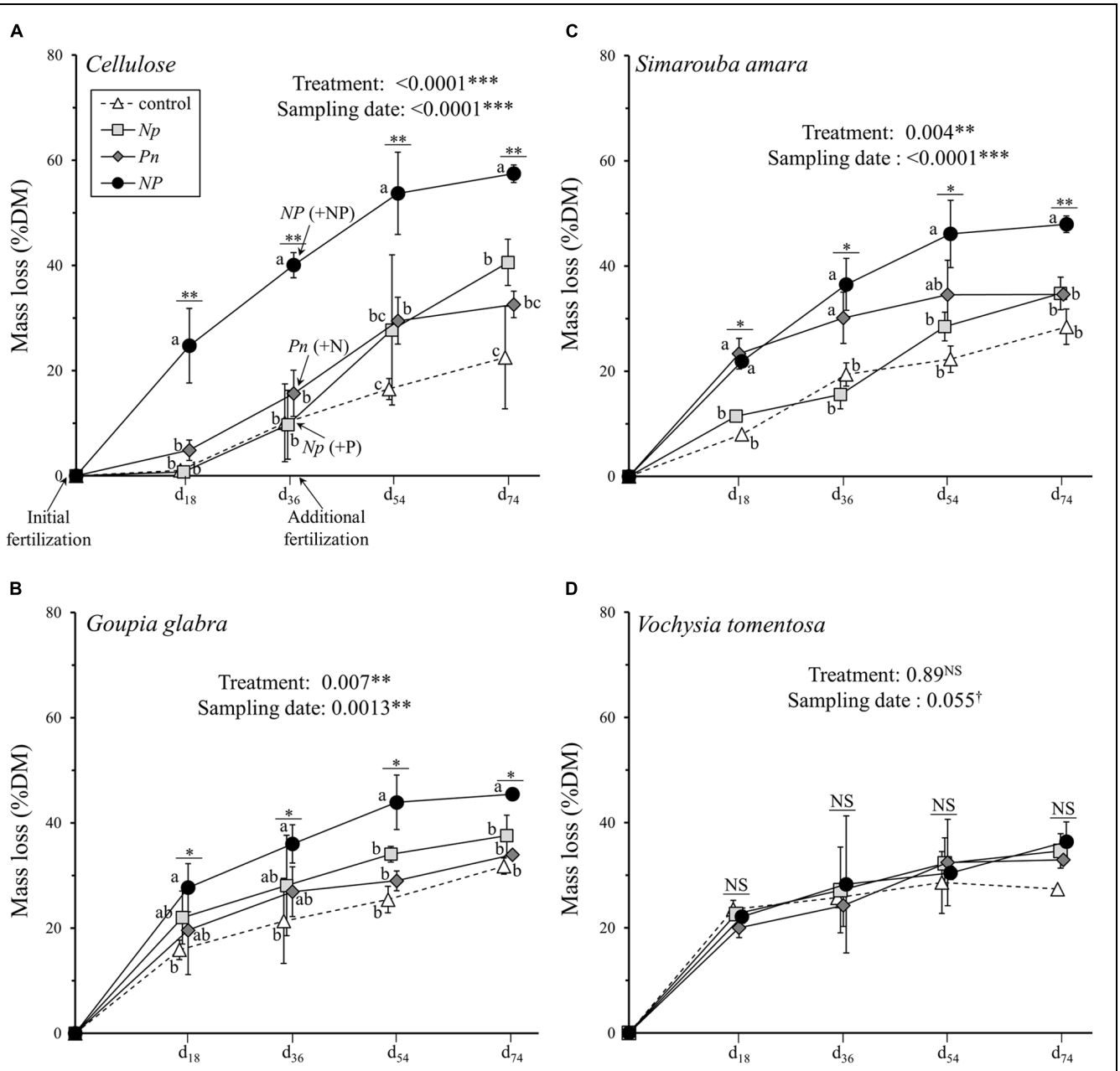

FIGURE 1 | Mass loss of the four different substrates (mean ± SD) as a function of sampling date. (A) cellulose, **(B)** *Goupia glabra*, **(C)** *Simarouba amara*, **(D)** *Vochysia tomentosa*. Fertilization treatments are shown with distinct symbols and curves (control: open triangle, dotted line; *NP*: black circles, solid line; *Np*: light gray square, solid line; *Pn*: dark gray diamond, solid line). Microcosms received the first dose of fertilizer (N, P, or NP) at d_0, and the second dose of fertilizers (i.e., P, N, or NP respectively) just after d_{36} sampling. Stars indicate significant differences in the mass loss for each date considered separately, followed by a *post hoc* Tukey-HSD test ($\alpha = 0.05$), with different letters indicating contrasted effects among the different fertilization treatments.

DISCUSSION

In line with the increasing number of studies demonstrating that the combined NP additions increase ecosystem processes more than P or N added singly (e.g., Vitousek et al., 2010), we found an overall stronger stimulation of substrate mass loss and litter microbial activity with a simultaneous NP supply compared to a temporally separated N and P addition. The fact that N and P together constrain the activity of microbial communities was particularly clear for litter SIR rates that on average varied sevenfold between the control and the *NP* treatments throughout the incubation period (**Figure 2**). These results are in line with previous studies showing that the decomposition of labile C substrates (Nottingham et al., 2012, 2015) or more complex leaf litter (Hobbie and Vitousek, 2000; Barantal et al., 2012; Fanin et al., 2012) were predominantly limited by concomitant

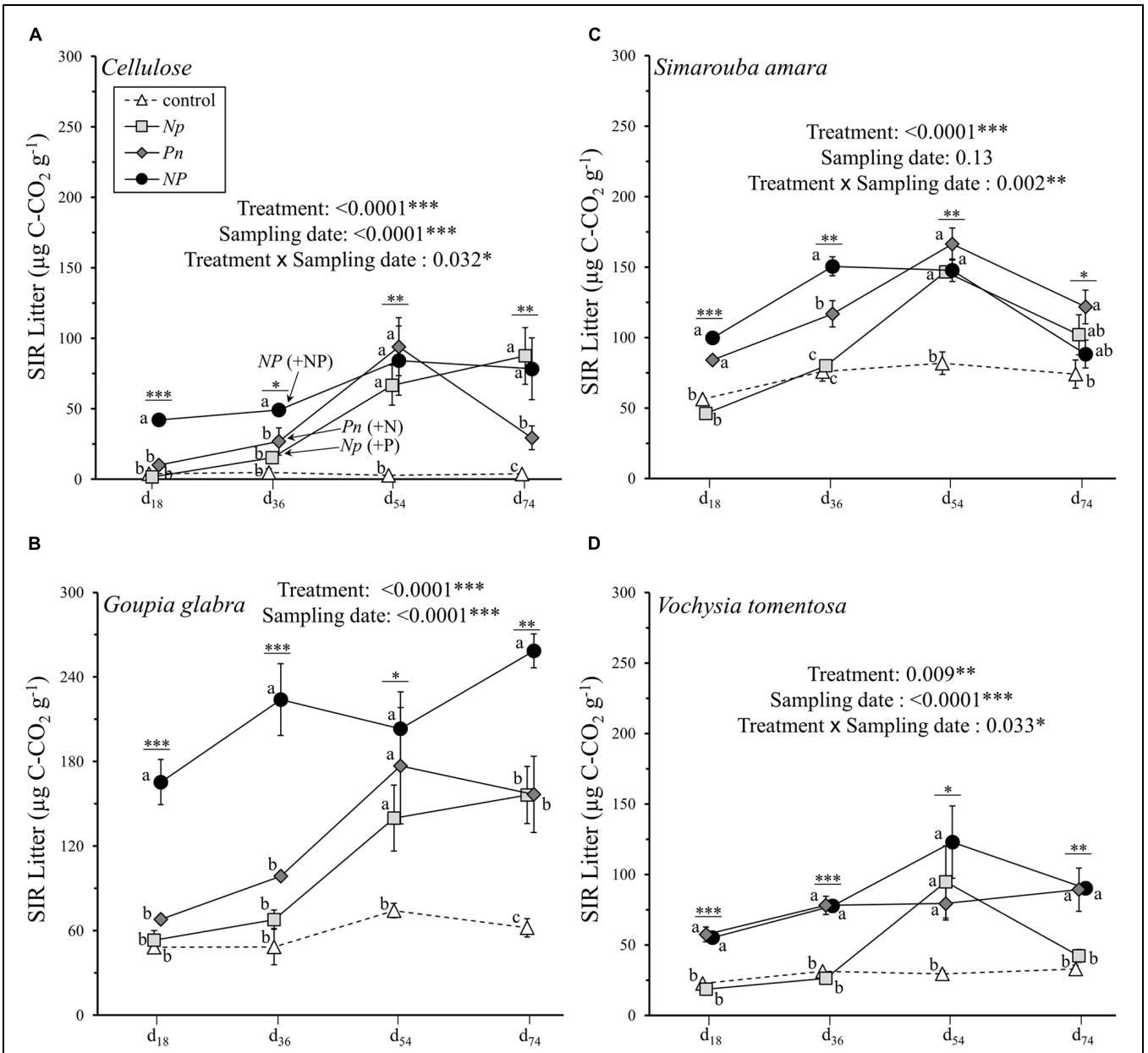

FIGURE 2 | Litter SIR rates of the four different substrates (mean ± SD) as a function of sampling date. (A) cellulose, **(B)** *Goupia glabra*, **(C)** *Simarouba amara*, **(D)** *Vochysia tomentosa*. Fertilization treatments are shown with distinct symbols and curves (control: open triangle, dotted line; *NP*: black circles, solid line; *Np*: light gray square, solid line; *Pn*: dark gray diamond, solid line). Stars indicate significant differences in the mass loss for each date considered separately, followed by a *post hoc* Tukey-HSD test ($\alpha = 0.05$), with different letters indicating contrasted effects among the different fertilization treatments.

NP additions. Because the stoichiometry of N and P supply and demand is generally in close balance in most terrestrial ecosystems (Vitousek et al., 2010), N is rarely available in great excess relative to P, and a slight addition of P can rapidly generate a N limitation or *vice-versa*: N and P limitation may thus alternate in multiple incremental steps, ultimately producing a synergistic effect when both nutrients are added together (Davidson and Howarth, 2007). In contrast to the 'Liebig world' view, which states that the nutrient in the shortest supply will be limiting, we demonstrate here that both elements can constrain litter decomposition and are required by microbial communities for maintaining their activity.

Beyond evaluating the effects of simultaneous NP additions, we addressed how the temporal variability in N and P availability affects litter decomposition and the activity of decomposer organisms. As expected following our first hypothesis, the responses of microbial parameters to a temporally separated N and P addition varied strongly among litter substrates (**Table 2**). We found that P supply showed a higher effect on SIR rates for the litter species with the highest soluble N content (i.e.,

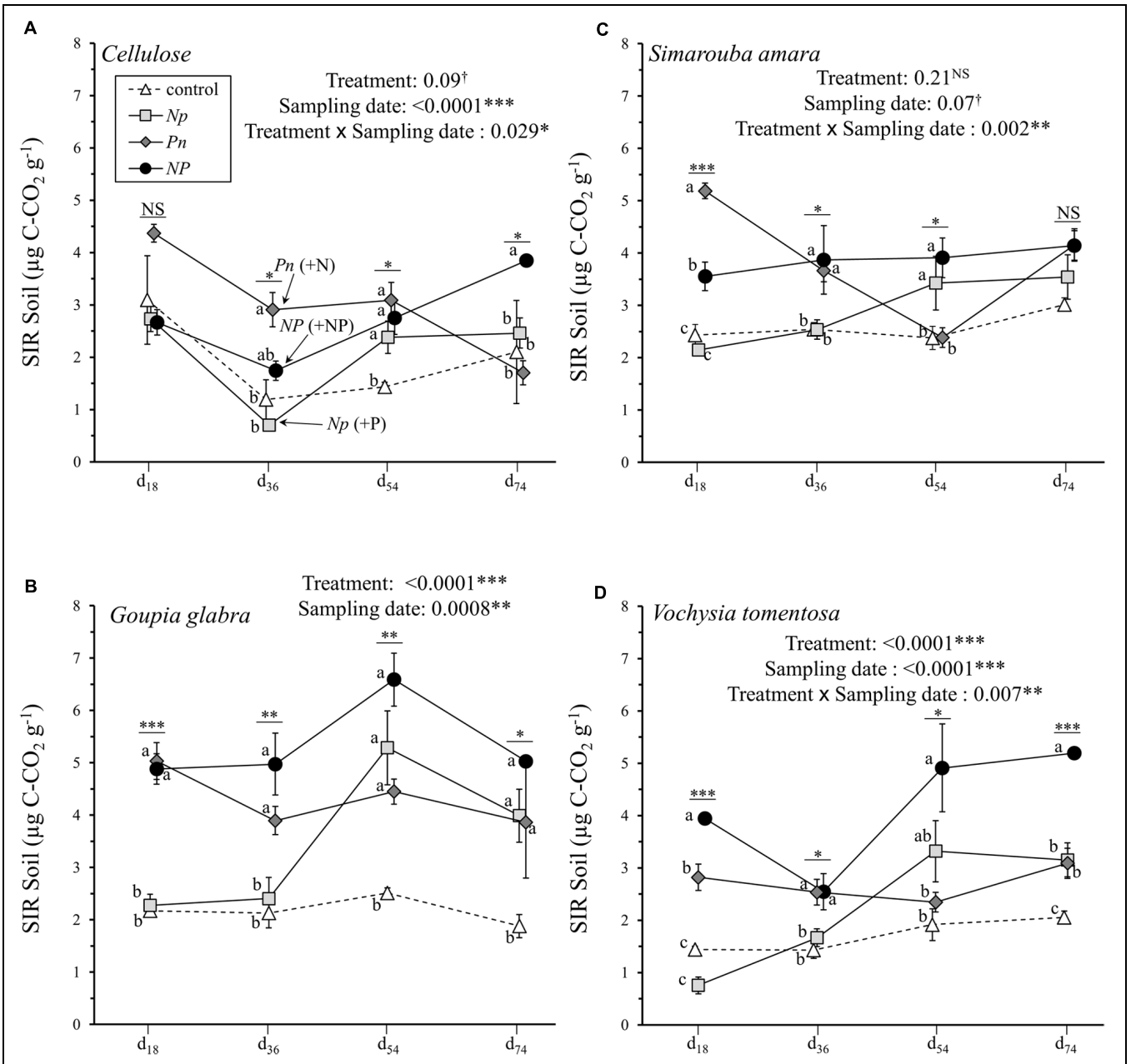

FIGURE 3 | Soil SIR rates of the four different substrates (mean ± SD) as a function of sampling date. (A) cellulose, **(B)** *Goupia glabra*, **(C)** *Simarouba amara*, **(D)** *Vochysia tomentosa.* Fertilization treatments are shown with distinct symbols and curves (control: open triangle, dotted line; *NP*: black circles, solid line; *Np*: light gray square, solid line; *Pn*: dark gray diamond, solid line). Stars indicate significant differences in the mass loss for each date considered separately, followed by a *post hoc* Tukey-HSD test ($\alpha = 0.05$), with different letters indicating contrasted effects among the different fertilization treatments.

S. amara and *V. tomentosa*), whereas the respective effects of N or P additions were similar when these two elements are relatively well balanced in litter leachates (i.e., *G. glabra*), or when they are completely absent (i.e., cellulose) (**Figure 2**). These results suggest that the relative imbalance between N and P in litter leachates can predict relatively well which of these two elements will be limiting during the initial phase of decomposition and may thus be used as a general indicator of nutritional constraints regulating the microbial activity. In other words, because the timing of nutrient availability in a given ecosystem can vary, depending for example on seasonal differences such as dry *versus* wet season in tropical forests (e.g., Turner et al., 2015), considering N and P in the soluble fraction of plant leaf litter may contribute to the understanding of temporal variability in soil processes.

Interestingly, after adding the second nutrient in the middle of the course of the experiment (i.e., N to the *Pn* treatment, and P to the *Np* treatment), SIR generally increased to similar rates as those observed in the *NP* treatment (**Figure 2**). In

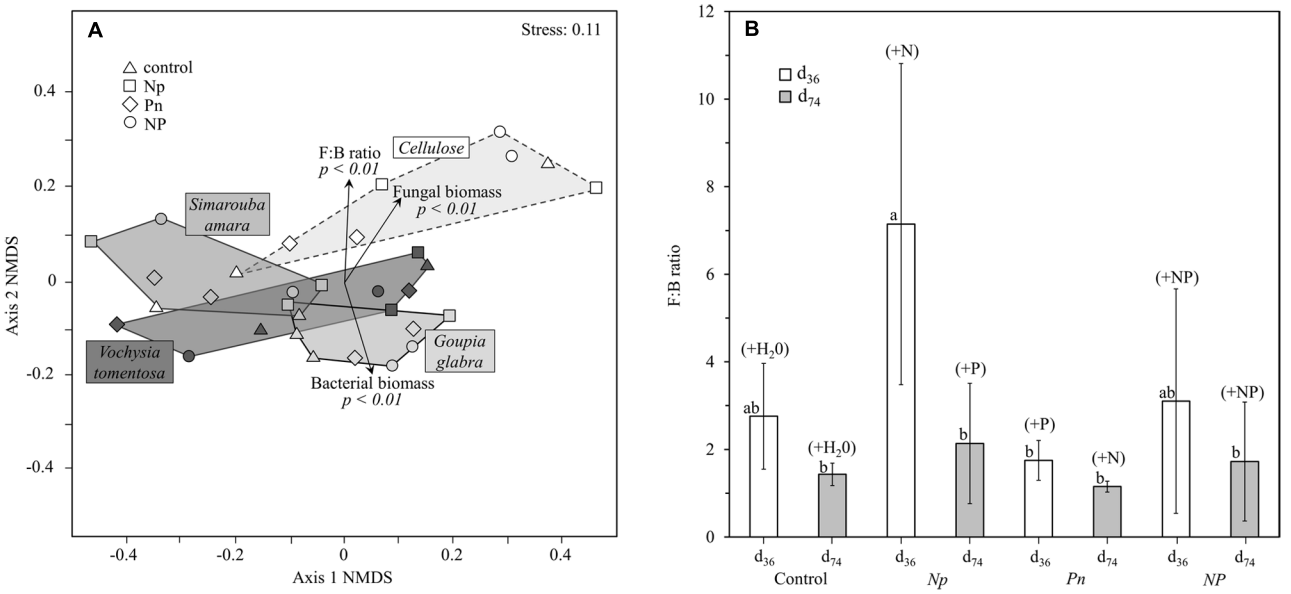

FIGURE 4 | Microbial community structure in leaf litter and cellulose: (A) NMDS ordination of microbial community composition by substrate identity, (B) fungi:bacteria ratio across all substrate types (except cellulose) as a function of the different fertilization treatments at d_{36} and d_{74}. NMDS was based on Bray–Curtis distances of 23 PLFA markers. With increasing distance between two points the community structure becomes more dissimilar. Vectors represent the proportion of the main microbial groups (bacteria, fungi) and their ratio (fungi:bacteria), pointing in the direction of higher abundances or a higher fungi:bacteria ratio.

contrast to our second hypothesis, the similar responses of microbial processes regardless of whether N or P was added first suggest that the order in relative N- and P-availability does not determine final microbial process rates, and confirms that litter decomposition and microbial activity are limited by multiple elements in interaction (Townsend et al., 2011; Fanin et al., 2015). In parallel, we observed a lower relative abundance of bacteria compared to fungi in response to N fertilization, in line with our expectation of enhanced fungal biomass following N additions (Koranda et al., 2014). Such a shift in the fungi to bacteria ratio was reversed following further addition of P later in the *Np* treatment (**Figure 4**), suggesting

TABLE 3 | Permutational multivariate ANOVA evaluating the role of substrate identity, fertilization treatment and sampling dates on microbial community structure in (A) litter, and (B) soil.

	df	SS	MS	F.model	*p-value*	r^2
(A) Litter community structure						
Substrate identity	3	0.84	0.28	13.0	0.001	0.421
Fertilization treatment	3	0.13	0.04	2.0	0.002	0.064
Sampling date	1	0.08	0.08	3.9	0.035	0.042
Substrate identity × Fertilization treatment	9	0.27	0.03	1.4	0.002	0.138
Fertilization treatment × Sampling date	3	0.07	0.02	1.1	0.001	0.201
Substrate identity × Sampling date	3	0.40	0.13	6.2	0.373	NS
Residuals	9	0.19	0.02			0.097
(B) Soil community structure						
Substrate identity	3	0.14	0.05	1.9	0.31	NS
Fertilization treatment	3	0.05	0.0.2	0.7	0.29	NS
Sampling date	1	0.18	0.18	7.2	0.002	0.187
Substrate identity × Fertilization treatment	9	0.17	0.02	0.8	0.59	NS
Fertilization treatment × Sampling date	3	0.10	0.03	1.4	0.032	0.107
Substrate identity × Sampling date	3	0.09	0.03	1.2	0.33	NS
Residuals	9	0.22	0.02			0.235

Permutational multivariate ANOVAs were performed to examine the relative influence of litter material, fertilization treatment, and time, as well as interactions between the three explanatory variables in accounting for the variation in microbial community composition over the course of the incubation experiment.

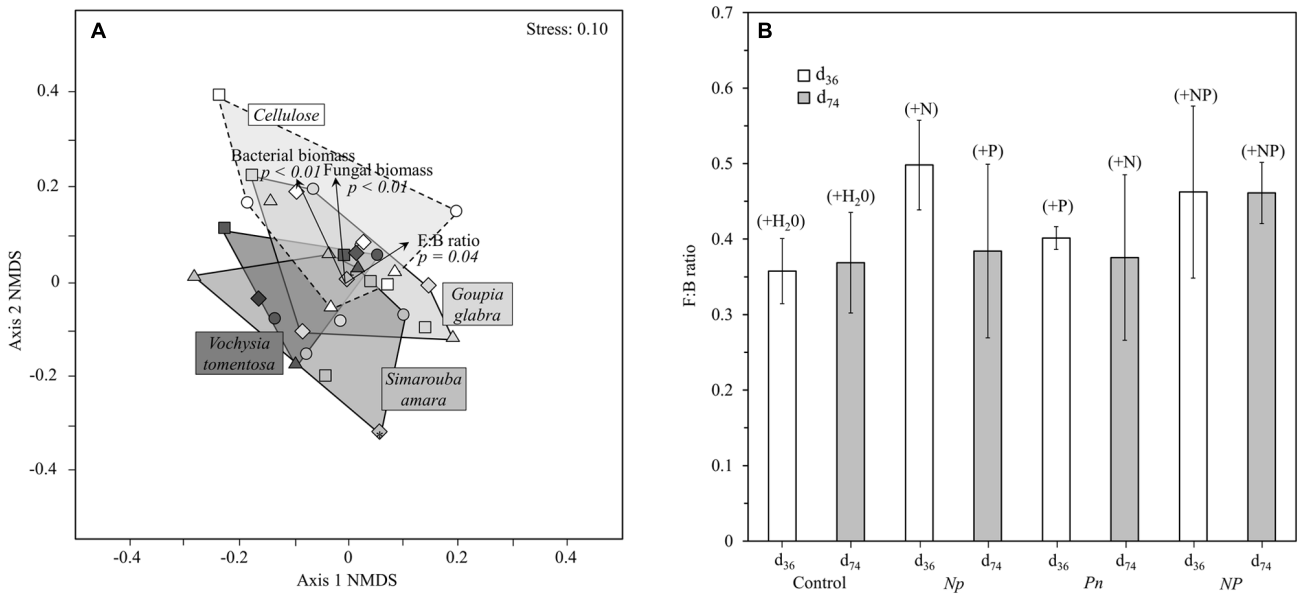

FIGURE 5 | Microbial community structure in the soil: (A) NMDS ordination of microbial community composition by substrate, (B) fungi:bacteria ratio across all substrate types (except cellulose) as a function of the different treatments at d_{36} and d_{74}. See **Figure 4** for further details.

that P is a more critical element for the fast-growing bacterial community compared to the relatively slower growing fungal community (Güsewell and Gessner, 2009). Thus, although the PLFA results should be interpreted with caution because of lack of replication, they suggest that any change in the relative availability of N and P can substantially alter the fungi:bacteria ratio, with bacteria dominating at low N: P ratios as it has been observed previously (Krashevska et al., 2010; Fanin et al., 2013).

Furthermore, the difference in the effect size between litter mass loss and litter SIR after simultaneous N and P additions indicate that the addition of a labile C source during SIR measurement may have also masked a potentially non-negligible C co-limitation on litter decomposition. For instance, the *NP* effect on litter mass loss was the strongest for cellulose (relatively accessible C substrate), intermediate for *G. glabra* or *S. amara* (both litter species relatively rich in DOC), and null for *V. tomentosa* (DOC-poor litter) (**Figure 1**). These results highlight that C availability may modulate the *NP* responses during litter decomposition, confirming that litter C leachates provide the microorganisms with the required energy to efficiently use available nutrients (Cleveland et al., 2006; Fanin et al., 2012). Accordingly, the community structure of microbial decomposers was more dependent on substrate identity than on fertilization treatments (**Table 3**). Cellulose decomposition appears to be mainly driven by fungi, while the leachate C-rich *G. glabra* leaf litter supported the highest proportion of bacteria relative to fungi of all the substrates. Such a C control on microbial community was even stronger in the soil, where substrate identity explained almost three times as much variation in soil SIR (59.4%) than did the fertilization treatments (21.5%) (**Table 2**). The fact that the nature of substrate leachates reaching the soil has a greater impact on soil microbial communities than nutrient fertilization is a rather surprising result (**Figure 5**). However, in line with our finding, Heuck et al. (2015) recently demonstrated that even in P-poor soils, the microbial biomass was primarily limited by C availability, suggesting that P is only secondarily limiting. Collectively, these results suggest that soil microorganisms are strongly limited by easily accessible C compounds originating from fresh litter material rather than by nutrients. C rather than nutrient limitation can in part be a consequence of the better balanced soil C: N: P stoichiometry compared to leaf litter with much wider C: N: P (Fanin et al., 2014; Mooshammer et al., 2014b). Perhaps even more important is the permanent high energy requirement of microorganisms in tropical soils with constantly high temperatures compared to soils at higher latitudes, which may reinforce C limitation in tropical rainforests (Hättenschwiler et al., 2011).

In summary, our study provides clear evidence that shifts in the relative availability of N and P through time regulate the structure and activity of the microbial decomposer community. Collectively, our data suggest that litter mass loss, microbial respiration and microbial community structure can vary temporally along with shifts in the relative availability of nutrients. These changes are rapid and reversible in response to changing nutrient availability. In fact, when both N and P are in excess in the environment, the order in the relative N and P availability did not determine final microbial process rates. The impact of successive N and P additions depended on the identity of decomposing litter material and their nutrient and C status of the soluble fraction, suggesting intimate interactions between exogenous nutrient availability and substrate-specific C

and nutrient leachates. Hence, the interactive effects of exogenous N and P availability depend in addition on litter-derived labile C with an even stronger impact on microbial communities in the soil compared to those in the litter layer. The consequences of N and P pulses on microbial communities and their activities in nutrient-poor tropical forests therefore depend to a large extent on litter-specific C quality, and thus, on tree species composition.

AUTHOR CONTRIBUTIONS

NiF formulated the idea, NiF and NaF established the analytical methods, conceived and designed the experiment, PS and NiF performed the analyses and collected the data, NiF and NaF analyzed data, NiF, NaF, and SH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Allison, S., and Vitousek, P. (2005). Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biol. Biochem.* 37, 937–944. doi: 10.1016/j.soilbio.2004.09.014

Bååth, E. (2003). The use of neutral lipid fatty acids to indicate physiological conditions of soil fungi. *Microb. Ecol.* 45, 373–383. doi: 10.1007/s00248-003-2002-y

Barantal, S., Schimann, H., Fromin, N., and Hättenschwiler, S. (2012). Nutrient and carbon limitation on decomposition in an Amazonian moist forest. *Ecosystems* 15, 1039–1052. doi: 10.1007/s10021-012-9564-9

Cleveland, C. C., and Liptzin, D. (2007). C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* 85, 235–252. doi: 10.1007/s10533-007-9132-0

Cleveland, C. C., Reed, S. C., and Townsend, A. R. (2006). Nutrient regulation of organic matter decomposition in a tropical rain forest. *Ecology* 87, 492–503. doi: 10.1890/05-0525

Cleveland, C. C., and Townsend, A. R. (2006). Nutrient additions to a tropical rain forest drive substantial soil carbon dioxide losses to the atmosphere. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10316–10321. doi: 10.1073/pnas.0600989103

Davidson, E. A., and Howarth, R. W. (2007). Environmental science: nutrients in synergy. *Nature* 449, 1000–1001. doi: 10.1038/4491000a

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Fanin, N., Barantal, S., Fromin, N., Schimann, H., Schevin, P., and Hättenschwiler, S. (2012). Distinct microbial limitations in litter and underlying soil revealed by carbon and nutrient fertilization in a tropical rainforest. *PLoS ONE* 7:e49990. doi: 10.1371/journal.pone.0049990

Fanin, N., Fromin, N., Buatois, B., and Hättenschwiler, S. (2013). An experimental test of the hypothesis of non-homeostatic consumer stoichiometry in a plant litter-microbe system. *Ecol. Lett.* 16, 764–772. doi: 10.1111/ele.12108

Fanin, N., Hättenschwiler, S., Barantal, S., Schimann, H., and Fromin, N. (2011). Does variability in litter quality determine soil microbial respiration in an Amazonian rainforest? *Soil Biol. Biochem.* 43, 1014–1022. doi: 10.1016/j.soilbio.2011.01.018

Fanin, N., Hättenschwiler, S., and Fromin, N. (2014). Litter fingerprint on microbial biomass, activity, and community structure in the underlying soil. *Plant Soil* 379, 79–91. doi: 10.1007/s11104-014-2051-7

Fanin, N., Hättenschwiler, S., Schimann, H., and Fromin, N. (2015). Interactive effects of C, N and P fertilization on soil microbial community structure and function in an Amazonian rain forest. *Funct. Ecol.* 29, 140–150. doi: 10.1111/1365-2435.12329

Frostegård, A., and Bååth, E. (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol. Fertil. Soils* 22, 59–65. doi: 10.1007/BF00384433

Galloway, J., Levy, H., and Kashibhatla, P. (1994). Year 2020 – Consequences of population-growth and development on deposition of oxidized nitrogen. *AMBIO* 23, 120–123.

Güsewell, S., and Gessner, M. O. (2009). N: p ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. *Funct. Ecol.* 23, 211–219. doi: 10.1111/j.1365-2435.2008.01478.x

Hartman, W. H., and Richardson, C. J. (2013). Differential nutrient limitation of soil microbial biomass and metabolic quotients (qCO2): is there a biological stoichiometry of soil microbes? *PLoS ONE* 8:e57127. doi: 10.1371/journal.pone.0057127

Hättenschwiler, S., Aeschlimann, B., Couteaux, M. M., Roy, J., and Bonal, D. (2008). High variation in foliage and leaf litter chemistry among 45 tree species of a neotropical rainforest community. *New Phytol.* 179, 165–175. doi: 10.1111/j.1469-8137.2008.02438.x

Hättenschwiler, S., Coq, S., Barantal, S., and Handa, I. T. (2011). Leaf traits and decomposition in tropical rainforests: revisiting some commonly held views and towards a new hypothesis. *New Phytol.* 189, 950–965. doi: 10.1111/j.1469-8137.2010.03483.x

Heuck, C., Weig, A., and Spohn, M. (2015). Soil microbial biomass C: N: P stoichiometry and microbial use of organic phosphorus. *Soil Biol. Biochem.* 85, 119–129. doi: 10.1016/j.soilbio.2015.02.029

Hobbie, S. E., and Vitousek, P. M. (2000). Nutrient limitation of decomposition in Hawaiian forests. *Ecology* 81, 1867–1877. doi: 10.2307/177277

Kaiser, C., Franklin, O., Dieckmann, U., and Richter, A. (2014). Microbial community dynamics alleviate stoichiometric constraints during litter decay. *Ecol. Lett.* 17, 680–690. doi: 10.1111/ele.12269

Kaspari, M., Garcia, M. N., Harms, K. E., Santana, M., Wright, S. J., and Yavitt, J. B. (2008). Multiple nutrients limit litterfall and decomposition in a tropical forest. *Ecol. Lett.* 11, 35–43. doi: 10.1111/j.1461-0248.2007.01124.x

Keiblinger, K. M., Hall, E. K., Wanek, W., Szukics, U., Hammerle, I., Ellersdorfer, G., et al. (2010). The effect of resource quantity and resource stoichiometry on microbial carbon-use-efficiency. *FEMS Microbiol. Ecol.* 73, 430–440. doi: 10.1111/j.1574-6941.2010.00912.x

Koranda, M., Kaiser, C., Fuchslueger, L., Kitzler, B., Sessitsch, A., Zechmeister-Boltenstern, S., et al. (2014). Fungal and bacterial utilization of organic substrates depends on substrate complexity and N availability. *FEMS Microbiol. Ecol.* 87, 142–152. doi: 10.1111/1574-6941.12214

Krashevska, V., Maraun, M., Ruess, L., and Scheu, S. (2010). Carbon and nutrient limitation of soil microorganisms and microbial grazers in a tropical montane rain forest. *Oïkos* 119, 1020–1028. doi: 10.1111/j.1600-0706.2009.18169.x

Manzoni, S., Taylor, P., Richter, A., Porporato, A., and Ågren, G. I. (2012). Environmental and stoichiometric controls on microbial carbon-use efficiency in soils. *New Phytol.* 196, 79–91. doi: 10.1111/j.1469-8137.2012.04225.x

McGill, W., and Cole, C. (1981). Comparative aspects of cycling of organic C, N, S, and P through soil organic matter. *Geoderma* 26, 267–286. doi: 10.1016/0016-7061(81)90024-0

McGroddy, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

Mooshammer, M., Wanek, W., Hämmerle, I., Fuchslueger, L., Hofhansl, F., Knoltsch, A., et al. (2014a). Adjustment of microbial nitrogen use efficiency to carbon: nitrogen imbalances regulates soil nitrogen cycling. *Nat. Commun.* 5:3694. doi: 10.1038/ncomms4694

Mooshammer, M., Wanek, W., Zechmeister-Boltenstern, S., and Richter, A. (2014b). Stoichiometric imbalances between terrestrial decomposer communities and their resources: mechanisms and implications of microbial adaptations to their resources. *Front. Microbiol.* 5:22. doi: 10.3389/fmicb.2014.00022

Mooshammer, M., Wanek, W., Schnecker, J., Wild, B., Leitner, S., Hofhansl, F., et al. (2012). Stoichiometric controls of nitrogen and phosphorus cycling in decomposing beech leaf litter. *Ecology* 93, 770–782. doi: 10.2307/23213726

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., and Renella, G. (2003). Microbial diversity and soil functions. *Eur. J. Soil Sci.* 54, 655–670. doi: 10.1046/j.1351-0754.2003.0556.x

Nottingham, A. T., Turner, B. L., Chamberlain, P. M., Stott, A. W., and Tanner, E. V. J. (2012). Priming and microbial nutrient limitation in lowland tropical forest soils of contrasting fertility. *Biogeochemistry* 111, 219–237. doi: 10.1007/s10533-011-9637-4

Nottingham, A. T., Turner, B. L., Stott, A. W., and Tanner, E. V. J. (2015). Nitrogen and phosphorus constrain labile and stable carbon turnover in lowland tropical forest soils. *Soil Biol. Biochem.* 80, 26–33. doi: 10.1016/j.soilbio.2014.09.012

Okin, G., Mahowald, N., Chadwick, O., and Artaxo, P. (2004). Impact of desert dust on the biogeochemistry of phosphorus in terrestrial ecosystems. *Global Biogeochem. Cycles* 18:81. doi: 10.1029/2003GB002145

Reed, S. C., Vitousek, P. M., and Cleveland, C. C. (2011). Are patterns in nutrient limitation belowground consistent with those aboveground: results from a 4 million year chronosequence. *Biogeochemistry* 106, 323–336. doi: 10.1007/s10533-010-9522-6

Roy, J., Hättenschwiler, S., and Domenach, A. M. (2005). "Tree diversity and soil biology: a new research program in French Guyana," in *Tree Species Effects on Soils: Implications for Global Change*, eds D. Binkley and O. Menyailo (Dordrecht: Kluwer Academic), 337–348.

Sinsabaugh, R. L., Shah, J. J. F., Findlay, S. G., Kuehn, K. A., and Moorhead, D. L. (2015). Scaling microbial biomass, metabolism and resource supply. *Biogeochemistry* 122, 175–190. doi: 10.1007/s10533-014-0058-z

Sterner, R., and Elser, J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Strickland, M. S., and Rousk, J. (2010). Considering fungal: bacterial dominance in soils: methods, controls and ecosystem implications. *Soil Biol. Biochem.* 42, 1385–1395. doi: 10.1016/j.soilbio.2010.05.007

Sundqvist, M. K., Liu, Z. F., Giesler, R., and Wardle, D. A. (2014). Plant and microbial responses to nitrogen and phosphorus addition across an elevational gradient in subarctic tundra. *Ecology* 95, 1819–1835. doi: 10.1890/13-0869.1

Townsend, A. R., Cleveland, C. C., Houlton, B. Z., Alden, C. B., and White, J. W. C. (2011). Multi-element regulation of the tropical forest carbon cycle. *Front. Ecol. Environ.* 9, 9–17. doi: 10.1890/100047

Turner, B. L., Yavitt, J. B., Harms, K. E., Garcia, M. N., and Wright, S. J. (2015). Seasonal changes in soil organic matter after a decade of nutrient addition in a lowland tropical forest. *Biogeochemistry* 123, 221–235. doi: 10.1007/s10533-014-0064-1

Vitousek, P. M., and Howarth, R. (1991). Nitrogen limitation on land and in the sea – How can it occur? *Biogeochemistry* 13, 87–115. doi: 10.1007/BF00002772

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. (2010). Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecol. Appl.* 20, 5–15. doi: 10.1890/08-0127.1

Walker, T. M., and Syers, J. K. (1976). The fate of phosphorus during pedogenesis. *Geoderma* 15, 1–19. doi: 10.1016/0016-7061(76)90066-5

Wood, T., Lawrence, D., and Clark, D. (2005). Variation in leaf litter nutrients of a Costa Rican rain forest is related to precipitation. *Biogeochemistry* 73, 417–437. doi: 10.1007/s10533-004-0563-6

Yuan, Z. Y., and Chen, H. Y. H. (2009). Global trends in senesced-leaf nitrogen and phosphorus. *Global Ecol. Biogeogr.* 18, 532–542. doi: 10.1111/j.1466-8238.2009.00474.x

Zelles, L. (1999). Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol. Fertil. Soils* 29, 111–129. doi: 10.1007/s003740050533

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Distinctive Tropical Forest Variants Have Unique Soil Microbial Communities, But Not Always Low Microbial Diversity

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There has been little study of whether different variants of tropical rainforest have distinct soil microbial communities and levels of diversity. We compared bacterial and fungal community composition and diversity between primary mixed dipterocarp, secondary mixed dipterocarp, white sand heath, inland heath, and peat swamp forests in Brunei Darussalam, Northwest Borneo by analyzing Illumina Miseq sequence data of 16S rRNA gene and ITS1 region. We hypothesized that white sand heath, inland heath and peat swamp forests would show lower microbial diversity and relatively distinct microbial communities (compared to MDF primary and secondary forests) due to their distinctive environments. We found that soil properties together with bacterial and fungal communities varied significantly between forest types. Alpha and beta-diversity of bacteria was highest in secondary dipterocarp and white sand heath forests. Also, bacterial alpha diversity was strongly structured by pH, adding another instance of this widespread pattern in nature. The alpha diversity of fungi was equally high in all forest types except peat swamp forest, although fungal beta-diversity was highest in primary and secondary mixed dipterocarp forests. The relative abundance of ectomycorrhizal (EcM) fungi varied significantly between forest types, with highest relative abundance observed in MDF primary forest. Overall, our results suggest that the soil bacterial and fungal communities in these forest types are to a certain extent predictable and structured by soil properties, but that diversity is not determined by how distinctive the conditions are. This contrasts with the diversity patterns seen in rainforest trees, where distinctive soil conditions have consistently lower tree diversity.

Keywords: biodiversity, microbial communities, soil pH, Southeast Asia, tropical rainforest

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INTRODUCTION

Ecologists are fascinated by patterns in the biodiversity of tropical rainforests, including how and why communities differ from one another, and why diversity is greater in some places than others. In terms of known biodiversity, Southeast Asian tropical rainforests are one of the most diverse terrestrial ecosystems on Earth (Corlett, 2014), with numerous poorly studied habitat types within them. The major equatorial rainforest types in Southeast Asia are mixed dipterocarp forest (MDF), heath forest and peat swamp forest (Whitmore, 1984). Heath and peat swamp forests occur on

acidic sandy and wet peaty soils, respectively, and support lower plant diversity compared to MDF (Bruenig and Droste, 1995; Davies and Becker, 1996; Slik et al., 2009). However, compared to macro-organisms, the community composition and diversity of microorganisms in these rainforest habitats is largely unknown.

Soil microorganisms constitute the largest proportion of the world's biodiversity and are important to terrestrial ecosystem functioning (Prosser, 2012). Thus, understanding their biodiversity patterns and the major divers of these patterns in natural habitats may be important for prediction of ecosystem responses to a changing environment (Jing et al., 2015). Previous studies of tropical soils have indicated that land use changes in tropical regions influence soil microbial communities, which are mainly driven by changes in soil chemical properties (Jesus et al., 2009; Tripathi et al., 2012; Lee-Cruz et al., 2013; Rodrigues et al., 2013; Kerfahi et al., 2014). Soil pH is becoming recognized as one of the most import drivers of microbial community structure and diversity in tropical soils at various scales (Jesus et al., 2009; Tripathi et al., 2014). There have also been some studies which compared the soil microbial community composition and diversity in different rainforest habitat types (Satrio et al., 2009; Araujo et al., 2012; Miyashita et al., 2013; Pacchioni et al., 2014; Pupin and Nahas, 2014). However, most of these studies were concentrated in Neotropical regions.

The present study concentrates on variation in soil bacterial and fungal community composition and diversity in several different types of lowland tropical rainforest habitat within Brunei Darussalam, Northwest Borneo, in Southeast Asia. The MDF forests dominate the lowland forests of Borneo (Ashton, 1988; Slik et al., 2003, 2009), whereas tropical heath and peat swamp forests are relatively distinctive habitats compared to MDF forests in Borneo in terms of species composition and diversity (Brünig, 1974; MacKinnon, 1996; Cannon and Leighton, 2004). Though the above ground diversity is well-studied in these various rainforest types, it is still unclear whether different rainforest habitats have distinct microbial community composition and diversity, analogous to the distinct plant community composition and diversity levels of these habitats (Bruenig and Droste, 1995; Davies and Becker, 1996).

The present study was conducted in Brunei Darussalam, Northwest Borneo, in Southeast Asia. Across Brunei, the major rainforest types are MDF primary, MDF secondary and peat swamp forests, with smaller scattered areas of heath forests (Whitmore, 1984). This concentration of a range of different rainforest types in close proximity provides an opportunity to study the soil microbial community composition and diversity under common climatic conditions while also diminishing the potential effect of dispersal limitation, meaning that detected differences can most likely be ascribed to differences in the soil and plant community only. We used 16S rRNA gene and ITS1 region amplicon sequencing using Illumina MiSeq platform to address the following questions:

(1) How do different rainforest habitats influence the OTU composition of soil bacteria and fungi, and what are the major soil properties linked to bacterial and fungal community structure?

(2) What are the dominant higher level bacterial and fungal taxa in each rainforest habitat type, and how does their relative abundance vary with respect to different rainforest habitats?

(3) How does the alpha and beta-diversity of bacteria and fungi vary across different rainforest habitats?

We hypothesized that white sand heath, inland heath and peat swamp forests would show lower alpha- and beta-diversity of bacteria and fungi, with relatively distinct microbial communities compared to MDF primary and secondary forests due to distinctive conditions of these environments.

MATERIALS AND METHODS

Site Description and Sample Collection

Five different lowland tropical rainforest types in Brunei Darussalam, Northwest Borneo were selected for this study (**Figure 1**). These forest types were MDF primary, mixed dipterocarp secondary, white sand heath, inland heath, and peat swamp forests. The MDF primary forest is dominated by large tree species in the family Dipterocarpaceae and the forest structure is complex and multi-layered. The sampled MDF secondary forests were aged around 60 years (Davies and Becker, 1996). Previously, the secondary forest sampling area was covered with primary forest (Davies and Becker, 1996). MDF secondary forest is characterized by similar plant species composition to the MDF primary forest, but differing by the dominance of pioneer tree species such as *Macaranga*, *Vitex,* and *Dillenia* species. The secondary MDF is also has a more open structure, consisting of a complex mosaic of near- mature and regenerating forest patches with contrasting plant compositions and micro-climates. The white sand and inland heath forests differ considerably from MDF forest both in plant species and structure (having a low and uniform single-layered canopy with dense undergrowth full of shrubs, herbs, pitcher plants, etc.). The main difference between the two heath forest types we sampled is that inland heath forest has low drainage capacity compared to white sand heath forest, which means that the white sand heath forest is being more susceptible to drought, while the inland heath forest can be flooded for part of the year. The peat swamp forest sampled in this study is dominated by a single canopy species of even aged/sized trees of *Shorea albida* (Dipterocarpaceae), while general plant diversity is much lower than in MDF, although the overall forest structure can be quite similar.

Field sampling was carried out during the month of June 2014, during a time with characteristic climate conditions in which afternoon rainstorms occurred about every other day (Becker, 1992). Brunei has a seasonal climate, with two drier periods February/March and July/August (Becker, 1992), and a mean annual rainfall above 2300 mm (David and Sidup, 1996). Three clusters of samples were taken in each forest type within a 3 km transect (**Figure 1**). Within each cluster, three quadrats (10 m × 10 m in size) were collected at least 30 m apart along a smaller scale linear transect (**Figure 1**). Each individual sample consisted of five pooled samples (each approximately 50 g from the four corners and one center point of the quadrat).

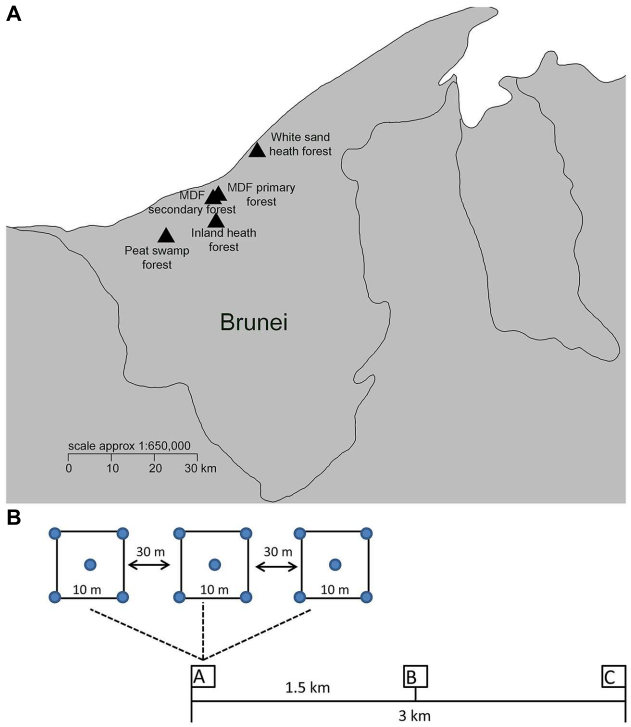

FIGURE 1 | (A) Soil sample locations of different forest types in Brunei. **(B)** Sampling scheme, three clusters of samples (designated as A–C) were taken in each forest type within a 3 km transect. Within each cluster, three quadrats (10 m × 10 m in size) were collected at least 30 m apart along a smaller scale linear transect. Soil collected from the four corners and center of the each quadrat was pooled to make one samples for DNA extraction and soil property analysis.

The top 10 cm of soil was collected in a sterile sampling bag after removing the litter layer. A total of 45 samples were collected from five different forest types (nine samples from each forest type). The collected soil samples were homogenized by sieving (2 mm sieve), and stored at −20°C until DNA extraction.

Soil Properties Analysis

Geographical co-ordinates and soil temperature at 5 cm depth were measured using a GPS device and a soil thermometer at each sampling quadrat during field sampling. Soil pH, gravimetric water content, organic matter content, total nitrogen and available phosphorus concentrations, and soil texture were measured at Universiti Brunei Darussalam using the standard methods (Allen, 1989). Total nitrogen content was determined by Kjeldahl method. Soil available phosphorus was extracted using Bray's reagent (0.025 M hydrochloric acid and 0.03 M ammonium fluoride), and the phosphorus concentration in the extracts was then determined using a UV-spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Soil organic matter content was determined after incineration in a muffle furnace at 550°C for 2 h, according to the methodology described by Allen (1989).

DNA Extraction, PCR, and Illumina Sequencing of 16S rRNA Gene and ITS1 Region

Soil DNA was extracted from each of the collected samples using the PowerSoil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following manufacturer's instructions, and DNA samples were sent to Macrogen Incorporated (Seoul, Korea) for PCR amplification and sequencing. The extracted DNA samples were amplified for V3 and V4 region of 16S rRNA gene using the primer pairs Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) for characterizing the bacterial communities (Herlemann et al., 2011). The fungal internal transcribed spacer (ITS) region 1 was amplified using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) primer pairs (White et al., 1990; Gardes and Bruns, 1993). The resulting 16S rRNA gene and ITS1 amplicons were sequenced using paired-end (2 × 300 nt) Illumina Miseq system (Illumina, USA).

Sequence Processing

The paired-end sequences of 16S rRNA gene and ITS1 amplicons were assembled using PANDAseq assembler (Masella et al., 2012). The initial sequence processing steps such as quality filtering and sequence alignment were performed using mothur (Schloss et al., 2009). The 16S rRNA gene sequences were aligned against a SILVA alignment[1]. Chimeric 16S rRNA gene and ITS1 sequences were identified using 'chimera.uchime' command implemented in mothur in *de novo* mode (Edgar et al., 2011), and removed. Taxonomic assignments of all the high quality 16S rRNA gene and ITS1 sequences were performed in mothur using the EzTaxon-e database[2] (Kim et al., 2012), and UNITE database (Abarenkov et al., 2010), respectively. To determine the ectomycorrhizal (EcM) fungi we matched the fungal taxonomic assignments with known EcM lineages (Tedersoo et al., 2010). The operational taxonomic units (OTUs) were assigned for 16S rRNA gene and ITS 1 sequences using mothur and QIIME implementation of UCLUST (Caporaso et al., 2010; Edgar, 2010), respectively, with a threshold of ≥97% sequence similarity. The entire singleton OTUs were removed prior to analysis. All the 16S rRNA gene and ITS1 sequences used in this study are deposited to metagenomic-RAST server (Meyer et al., 2008) under the project ID 14875[3].

Statistical Analysis

Prior to statistical analysis, a random subset of 3,352 and 4,207 sequences per sample was generated for 16S rRNA gene and ITS 1 sequences, respectively, to correct for the differences in number of reads. To assess the differences in soil properties among different forest types, we used analysis of variance (ANOVA) or Kruskal–Wallis tests for normal and non-normal data, respectively. Furthermore, parametric (Tukey's HSD test) or non-parametric (pairwise Wilcox test) *post hoc* tests were

[1]http://www.arb-silva.de/

[2]http://eztaxon-e.ezbiocloud.net/

[3]http://metagenomics.anl.gov/linkin.cgi?project=14875

used in case of significant results of ANOVA or Kruskal–Wallis tests, respectively. We used the Benjamini-Hochberg correction to assess pairwise comparisons ($P < 0.05$; Benjamini and Hochberg, 1995). A principal components analysis (PCA) was performed on the correlation matrix of soil properties data of each sample in Canoco 5.0 (Biometrics, Wageningen, The Netherlands). We used permutational multivariate analysis of variance (PerMANOVA, 'adonis' function in vegan R package) to test the effect of forest type on a Euclidean distance matrix of normalized soil properties data with 9999 random permutations.

Cluster analysis was performed on Bray–Curtis distance matrices of bacterial and fungal OTUs by using an unweighted pair group mean (UPGMA) algorithm implemented in the 'hclust' function of vegan R package (Oksanen et al., 2007). As 16S rRNA genes are suitable for phylogenetic analysis, a unweighted UniFrac distance matrix was also generated for bacteria (Lozupone et al., 2011). Bray–Curtis and unweighted UniFrac distance matrices were further visualized by non-metric multidimensional scaling (NMDS) plots. Furthermore, PerMANOVA was used to evaluate the effect of forest type on Bray–Curtis and unweighted UniFrac distance matrices with 9999 random permutations. To detect possible associations between bacterial and fungal community structure and soil properties, the vectors of significant soil properties ($P < 0.05$) were fitted onto ordination space using the 'envfit' function of the vegan R package with 999 random permutations.

The significant differences in composition and diversity of bacterial and fungal taxa in different forest types were analyzed by ANOVA or Kruskal–Wallis tests as described above. To test the relationship between soil properties and the relative abundance of dominant bacterial and fungal phyla, we used the Spearman rank correlation test. We performed linear regression analysis to test for differences in alpha-diversity (Shannon index) in relation to soil properties. We used the betadisper function of 'vegan' R package to assess the differences in beta-diversity among different forest types, and significance of this test was determined using 999 permutations.

RESULTS

Soil Properties among Forest Types

All the measured soil properties varied significantly among different forest types, except for total nitrogen and silt concentrations (**Table 1**). PCA of the different soil properties measured indicated that peat swamp forest sites were clearly distinct from other forest types (Supplementary Figure S1); however, sites from other forest types were not well-separated from each other (Supplementary Figure S1). The first two axis of the PCA explained about 71% of the total variance, with axis 1 and 2 explaining 51.6 and 19.4% of the total variance, respectively. The PerMANOVA analysis revealed a statistically significant effect of forest type on soil properties ($P < 0.001$, 9999 permutations).

Bacterial and Fungal Community Composition among Forest Types

The UPGMA clustering analysis based on Bray–Curtis distance showed that bacterial and fungal community compositions were largely separated by forest types (Supplementary Figure S2). The bacterial and fungal communities in white sand heath forest were most distinct from all other forest types (Supplementary Figure S2). Whereas, MDF primary and secondary forests had most similar bacterial and fungal community composition. However, in the MDF secondary forest there were two and four extreme bacterial and fungal communities, respectively. The bacterial communities of inland heath and peat swamp forests were at the same distance level to the MDF primary and secondary forest communities, whereas, bacterial communities in these forests were in turn at the same distance level to those in the white sand heath forest. In the case of fungi, inland heath forest communities are closer in composition to MDF forests than to peat swamp and white sand heath forests communities. The UPGMA clustering results were further corroborated by the NMDS ordination plot, which also showed that bacterial and fungal community compositions were segregated by forest type (**Figures 2A,B**). The PerMANOVA analyses indicated that forest type explained 36.1 and 37.8% variation in bacterial and fungal community composition, respectively ($P < 0.001$, 9999 permutations). The phylogenetic community composition of bacteria, based on unweighted UniFrac distance also displayed similar pattern as that of bacterial OTU composition (Supplementary Figure S3), and also significantly influenced by forest type (PerMANOVA, $P < 0.001$, 9999 permutations).

To further investigate the effect of soil properties on bacterial and fungal community structure, the vectors of environmental variables were fitted onto ordination space. The environmental fitting analysis indicated that of the measured soil properties, soil pH, organic matter content, gravimetric water content, available phosphorus, temperature, sand and clay content were strongly correlated with bacterial and fungal community structure (Supplementary Figure S3 and **Figures 4A,B**).

Dominant Bacterial and Fungal Taxa

A total of 150,840 good quality bacterial 16S rRNA gene sequences were obtained (3,352 randomly selected reads per sample). *Proteobacteria* was the most dominant bacterial phylum (40.6% of all bacterial sequences) followed by *Acidobacteria* (37.2%), *Planctomycetes* (7.1%), *Actinobacteria* (3.5%), *Verrucomicrobia* (3.4%), and *Chloroflexi* (2.9%; **Figure 3A**). Except *Planctomycetes*, the relative abundance of these phyla varied significantly ($P < 0.05$) among forest types (**Table 2**). For fungal ITS1 sequences, a total of 189,315 high quality sequences were obtained from 45 samples (4,207 randomly selected reads per sample). The most abundant fungal phylum detected across all samples was Ascomycota (54.1% of all fungal sequences) followed by Basidiomycota (15.4%), and 30.1% of the detected sequences were unclassified (**Figure 3B**). The relative abundance of these most abundant fungal phyla varied significantly in relation to different forest types (**Table 2**). The relative abundance of Ascomycota was higher in white sand and

TABLE 1 | Values of (mean ± SD) of soil properties in different forest types.

Soil properties	MDF primary forest	MDF secondary forest	White sand heath forest	Inland heath forest	Peat swamp forest
Temperature (°C)[2]	26.1 ± 0.4 b	27.8 ± 1.5 a	27.4 ± 0.7 a	26.2 ± 0.4 b	26.7 ± 0.3 a
Gravimetric water content (gg^{-1})[2]	0.24 ± 0.06 c	0.18 ± 0.02 c	0.26 ± 0.13 c	0.46 ± 0.14 b	2.95 ± 0.53 a
pH[1]	3.9 ± 0.2 b	4.3 ± 0.3 a	4.2 ± 0.3 ab	3.2 ± 0.2 c	3.4 ± 0.2 c
Organic matter (%)[2]	12.6 ± 3.5 c	10.4 ± 5.9 c	46.7 ± 11.9 b	45.1 ± 17.7 b	97.7 ± 0.6 a
Total nitrogen (mg g^{-1})[1]	16.1 ± 4.3 a	10.2 ± 5.5 a	17.2 ± 9.7 a	15.1 ± 13.2 a	9.2 ± 1.6 a
Available phosphorus (μg g^{-1})[2]	67.6 ± 2.8 b	68.5 ± 8.1 b	64.4 ± 2.6 b	64.1 ± 2.5 b	85.4 ± 7.7 a
Clay (%)[2]	7.9 ± 5.7 a	4.9 ± 2.5 ab	2.4 ± 2.3 bc	2.2 ± 0.9 c	11.6 ± 9.2 a
Silt (%)[2]	71.7 ± 6.3 a	75.3 ± 3.6 a	72.8 ± 3.5 a	73.6 ± 4.7 a	70.1 ± 9.9 a
Sand (%)[2]	20.4 ± 2.4 ab	19.8 ± 2.0 ab	24.7 ± 4.3 a	24.2 ± 5.3 a	18.1 ± 1.8 b

Means on the same row with different letters were significantly different ($P < 0.05$) based on Tukey's HSD test (1) or pairwise Wilcox test (2) followed by Benjamini-Hochberg correction for multiple comparisons.

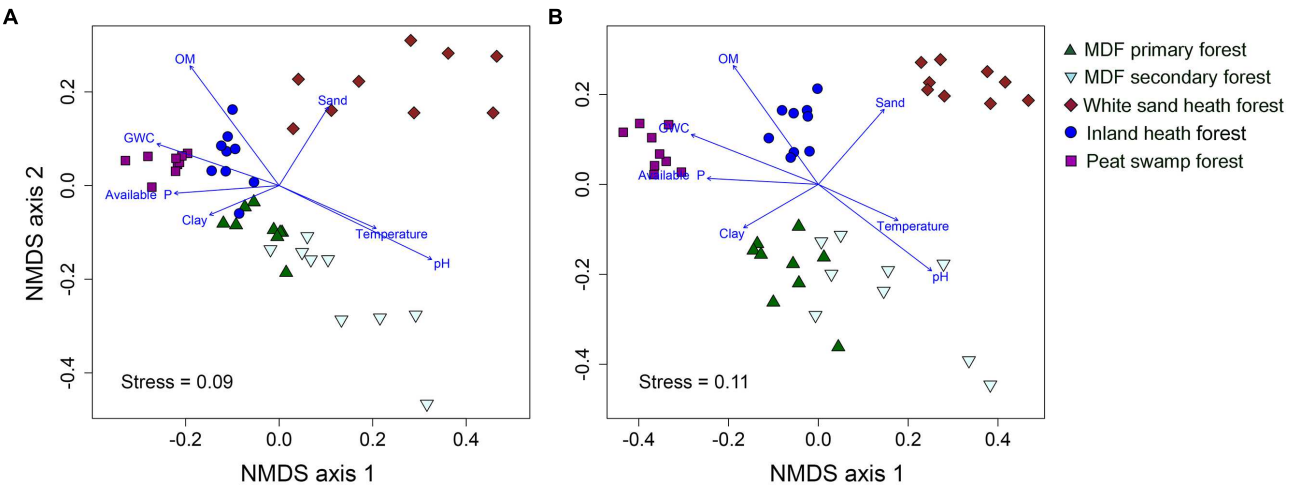

FIGURE 2 | Non-metric multidimensional scaling plot of (A) bacterial, and (B) fungal communities based on pairwise Bray–Curtis distances. A vector overlay of the significantly correlated variables is shown on the plot. GWC, gravimetric water content and OM, organic matter content.

inland heath forests (**Table 2**), whereas the relative abundance of Basidiomycota was higher in MDF primary and secondary forests (**Table 2**).

A total of 18,460 sequences belonged to known EcM fungal genera, representing around 9.7% of the total detected fungal sequences. The relative abundance of the detected EcM fungal genera varied significantly between forest types, with highest and lowest relative abundance observed in MDF primary forest and white sand heath forest, respectively ($P < 0.0001$; **Table 2**). The most abundant EcM fungal genus was *Russula* (78% of total EcM sequences), followed by *Amanita*, *Thelephora,* and *Tomentella*. The relative abundance of *Russula* also varied significantly between forest types, and showed similar pattern to that of total EcM fungi (Supplementary Table S1).

The relative abundance of *Proteobacteria*, *Acidobacteria*, and *Chloroflexi* was significantly correlated with gravimetric water content, soil pH, and organic matter content (**Table 3**). Whereas, the reactive abundance of *Planctomycetes* and *Actinobacteria* correlated with clay content (**Table 3**). Additionally, soil temperature and sand content was also found to be correlated with the relative abundance of *Acidobacteria* and *Actinobacteria*, respectively. The relative abundance of *Verrucomicrobia* was not correlated with any of the environmental variables measured (**Table 3**). The relative abundance of the most dominant fungal phylum Ascomycota was significantly correlated only with clay content, whereas the relative abundance of Basidiomycota the other dominant fungal phylum was significantly correlated with gravimetric water content and organic matter content.

The Alpha and Beta-Diversity of Bacteria and Fungi

The alpha-diversity index (OTU richness and Shannon index) of both bacteria and fungi also varied significantly among forest types (**Table 4**). The lowest average bacterial alpha diversity was observed in inland heath and peat swamp forest, whereas fungal alpha diversity was lowest in peat swamp forest only, although due to high variation in diversity values, considerable overlap in diversity existed between some forest types (**Table 4**). Bacterial Shannon diversity index correlated positively with soil temperature and pH (**Figure 4A**), whereas gravimetric water content and organic matter content of the soils displayed negative correlation with bacterial diversity indices (**Table 3**). Available

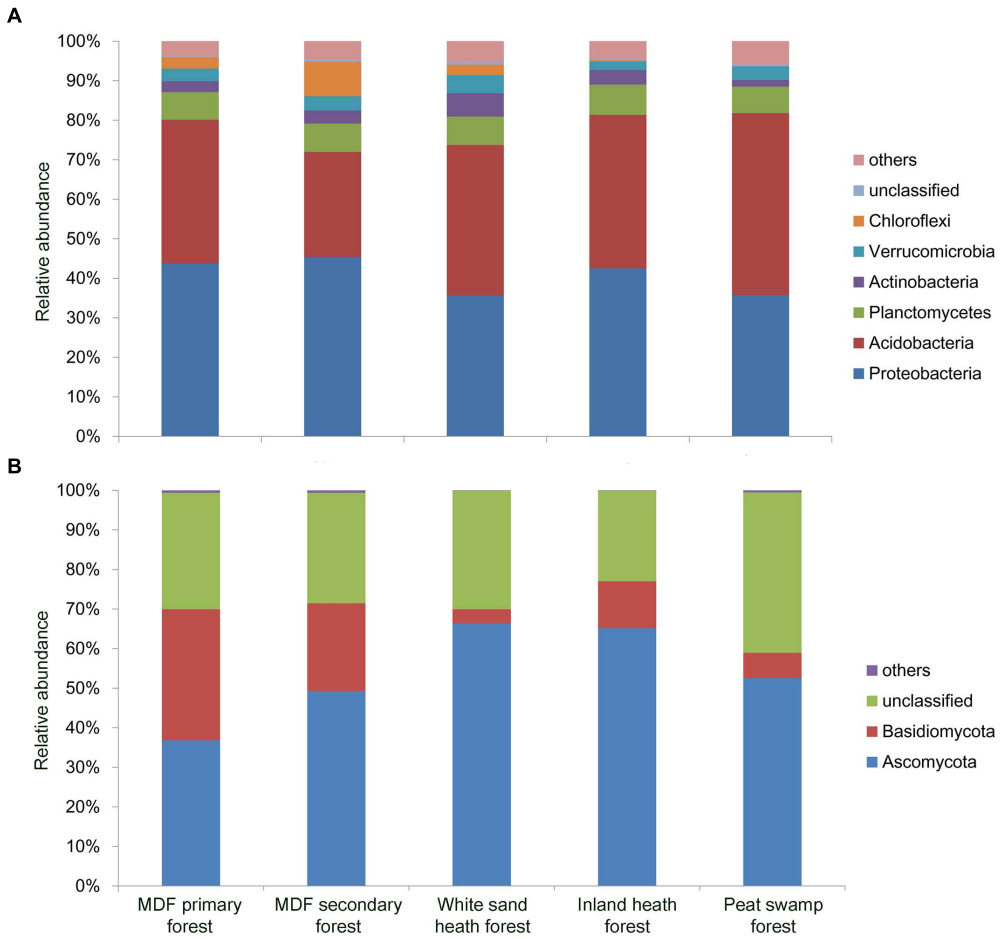

FIGURE 3 | Relative abundance of dominant (A) bacterial phyla observed in 16S rRNA gene sequences and (B) fungal phyla in ITS1 sequences in different forest types.

TABLE 2 | Comparison of relative abundance (mean ± SD) of dominant bacterial and fungal phyla, and EcM fungi among forest types.

Dominant taxa	MDF primary forest	MDF secondary forest	White sand heath forest	Inland heath forest	Peat swamp forest
Bacteria					
Proteobacteria[1]	43.8 ± 3.9 a	45.4 ± 5.5 a	35.6 ± 3.3 b	42.5 ± 4.3 a	35.8 ± 2.0 b
Acidobacteria[1]	36.3 ± 5.0 b	26.6 ± 4.3 c	38.1 ± 5.0 b	38.8 ± 6.7 b	46.0 ± 3.3 a
Planctomycetes[1]	7.0 ± 0.9 a	7.1 ± 1.5 a	7.2 ± 1.0 a	7.7 ± 1.5 a	6.7 ± 1.0 a
Actinobacteria[2]	2.8 ± 1.0 b	3.4 ± 1.5 b	5.9 ± 2.6 a	3.6 ± 2.1 b	1.7 ± 0.9 c
Verrucomicrobia[2]	3.2 ± 0.5 a	3.6 ± 1.5 a	4.6 ± 2.3 a	2.2 ± 0.5 b	3.5 ± 0.8 a
Chloroflexi[2]	2.9 ± 1.8 b	8.7 ± 6.1 a	2.6 ± 2.4 b	0.4 ± 0.5 c	0.1 ± 0.1 c
Fungi					
Ascomycota[1]	36.9 ± 18.1 b	49.3 ± 18.7 ab	66.3 ± 12.4 a	65.2 ± 16.6 a	52.6 ± 23.8 ab
Basidiomycota[1]	33.1 ± 17.7 a	22.2 ± 17.6 ab	3.7 ± 2.9 c	11.8 ± 11.8 bc	6.4 ± 3.9 bc
EcM fungi[2]	25.8 ± 18.0 a	12.8 ± 17.5 abc	0.1 ± 0.0 d	9.2 ± 11.1 b	0.9 ± 0.8 c

Different letters represent means that were significant different ($P < 0.05$) based on Tukey's HSD test (1) or pairwise Wilcox test (2) followed by Benjamini-Hochberg correction for multiple comparisons.

phosphorus and soil clay content were negatively correlated with the Shannon index of the fungi (**Figure 4B**), while sand content was found to be positively correlated with fungal Shannon index (**Table 3**). The Whittaker beta-diversity of bacterial and fungal communities, measured as the average distance of all samples to the centroid in each forest type varied significantly among forest types (**Figure 5**). The MDF secondary and white sand heath forests having highest bacterial beta-diversity, whereas MDF

TABLE 3 | Spearman rank correlations between soil properties and the relative abundance of dominant bacterial and fungal phyla, and alpha diversity indices.

Jamini	Temperature (°C)	GWC (gg^{-1})	pH	Organic matter (%)	Total nitrogen ($mg\ g^{-1}$)	Available phosphorus ($\mu g\ g^{-1}$)	Clay (%)	Sand (%)	Silt (%)
Bactetial phyla									
Proteobacteria	0.04	−0.6***	0.52***	−0.58***	0.24	−0.11	0.1	0.08	−0.03
Acidobacteria	−0.34*	0.61***	−0.66***	0.65***	−0.2	0.26	0.08	−0.11	−0.15
Planctomycetes	0.04	−0.14	0.23	0.04	0.26	−0.05	−0.44**	0.16	0.18
Actinobacteria	0.12	−0.21	0.11	−0.13	0.26	−0.59	−0.31*	0.38**	0.09
Verrucomicrobia	0.08	0.21	0.02	0.09	0.11	0.04	0.26	−0.2	−0.02
Chloroflexi	0.09	−0.52***	0.51***	−0.71***	0.17	−0.28	0.01	0.11	0.09
Fungal phyla									
Ascomycota	0.08	0.19	−0.11	0.3	−0.05	−0.21	−0.37*	0.26	0.1
Basidiomycota	−0.09	−0.44**	0.27	−0.51***	0.03	0.03	0.29	−0.09	−0.12
Alpha diversity bacteria									
OTU richness	0.49**	−0.45**	0.8***	−0.45**	0.12	−0.05	0.04	−0.07	0.18
Shannon index	0.51***	−0.45**	0.8***	−0.48**	0.11	−0.02	0.1	−0.07	0.19
Alpha diversity fungi									
OTU richness	−0.3	−0.28	0.13	−0.4*	0.15	−0.41**	−0.18	0.26	0.14
Shannon index	0.03	−0.25	0.25	−0.24	−0.03	−0.43**	−0.35*	0.44**	0.12

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 4 | The alpha diversity indices (OTU richness and Shannon index) of bacteria and fungi in different forest types.

	MDF primary forest	MDF secondary forest	White sand heath forest	Inland heath forest	Peat swamp forest
Bacteria					
OTU richness	619 ± 37 ab	703 ± 82 a	688 ± 93 a	510 ± 46 c	567 ± 44 bc
Shannon index	5.0 ± 0.1 bc	5.3 ± 0.3 a	5.3 ± 0.4 ab	4.7 ± 0.1 d	4.9 ± 0.1 cd
Fungi					
OTU richness	390 ± 94 a	361 ± 80 a	318 ± 55 ab	372 ± 73 a	243 ± 44 b
Shannon index	3.8 ± 0.9 ab	3.9 ± 0.9 ab	4.1 ± 0.5 a	4.1 ± 0.5 a	3.0 ± 0.6 b

Values with different letters were significantly different ($P < 0.05$) based on Tukey's HSD test followed by Benjamini-Hochberg correction for multiple comparisons.

primary and secondary forests had highest fungal beta-diversity (**Figure 5**).

DISCUSSION

Distinct Soil Conditions amongst the Different Forest Types

The soil conditions varied significantly among different forest types (**Table 1**), reflecting the broad scale mosaic of environments within the lowland forests of Brunei (Moran et al., 2000; Din et al., 2015). The primary dipterocarp forest was toward the more acidic end of the normal range of pH for lowland terra firme rainforest (about pH 3.7–5.5), with typical available P and total N content (Sukri et al., 2012). The secondary forest had somewhat higher pH, but similar available P and total N levels. Soil temperature in the secondary forest at the time of sampling was somewhat higher on average, likely due to the more open canopy allowing greater daytime heating of the soil surface. Organic matter content was higher in both white sand and inland heath forests compared to MDF primary and secondary forest. The water logged environment of heath forests with limited oxygen levels might lead to accumulation of organic matter content (Moran et al., 2000). The sand content of both heath forest types was similar to the MDF primary and secondary forests. Our own examination of these soils before analyses showed that the white quartz grain component of the soils in both these heath forest types was unusually fine grade at these sites, and probably it ended up classified as silt grade. The inland heath forest was very acidic and high in soil gravimetric water content, showing swampy conditions. Swampy areas in heath forests are quite common in this region (Moran et al., 2000; Din et al., 2015). The peat swamp forest soils were very acidic, with higher organic matter and available P contents than the other forest types, and its gravimetric water content was much higher – reflecting the high water table and the abundant spongy peat. The higher level of nutrients in peat swamp forest is not unusual, as the peat soil is generated from the accumulation of partially decayed organic matter due to water logged conditions with limited oxygen supply (Andriesse, 1988; Satrio et al., 2009).

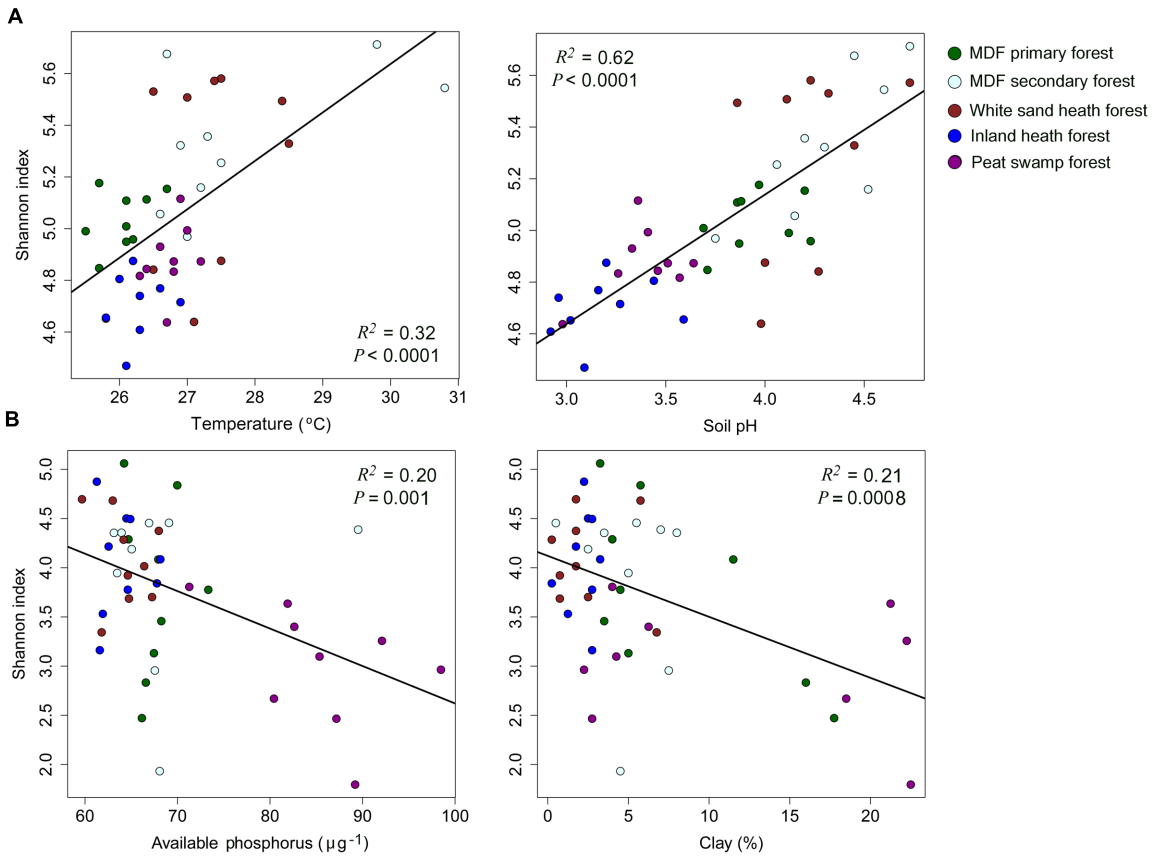

FIGURE 4 | The relationships between (A) bacterial, and (B) fungal Shannon index and soil properties with symbols coded by forest types. Linear regressions were used to test the correlation between Shannon index and soil properties.

Are There Distinct Microbial Communities on Different Rainforest Types?

The results of our comparison of soil bacterial and fungal communities across Brunei forests revealed that there are distinct community types in different types of rainforest. The community composition of fungi and bacteria in each forest type was significantly different from all of the others, but the most distinct community is that of the peat swamp forest. The distinct nature of these communities suggests that there is strong environmental and perhaps evolutionary selection for both bacterial and fungal OTUs better adapted in each environment. The clustering patterns of bacterial and fungal communities were very similar to one another, and influenced by soil pH, organic matter content, gravimetric water content, available phosphorus, temperature, sand and clay content. Previous studies on tropical soils have also shown that microbial community composition is influenced by variations in underlying soil properties due to land use change (Jesus et al., 2009; Tripathi et al., 2012, 2013; Lee-Cruz et al., 2013; Kerfahi et al., 2014).

The most abundant bacterial phyla detected across the samples were *Proteobacteria* and *Acidobacteria*, which is consistent with the results of previous studies on rainforest soils (Kanokratana et al., 2011; Tripathi et al., 2012; Lee-Cruz et al., 2013). The relative abundance of *Proteobacteria* was significantly lower in white sand heath and peat swamp forests, and this result could be due to restricted nutrient availability in oxygen-limited waterlogged environments of white sand heath peat swamp forests (Moran et al., 2000; Page et al., 2006). It has been shown that the relative abundance of major proteobacterial subphyla increases with nutrient additions (Leff et al., 2015). However, the relative abundance of *Acidobacteria* was significantly higher in peat swamp forests and negatively correlated with soil pH – a result which is to be expected as peat soils had very low pH, and most of the acidobacterial lineages are shown to dominate acidic soil environments (Jones et al., 2009). The relative abundance of *Actinobacteria* was highest in white sand heath forest, and positively correlated with the sand content of soil. These results are in agreement with previous observations that the members of phylum *Actinobacteria* are generally abundant in sandy forest soils (Russo et al., 2012; Pacchioni et al., 2014). The dominance of photosynthetic bacterial phylum *Chloroflexi* in secondary forests could be explained by that, due to more open canopy secondary forest soils are exposed to sunlight to a greater extent than other forest soils (Nacke et al., 2014).

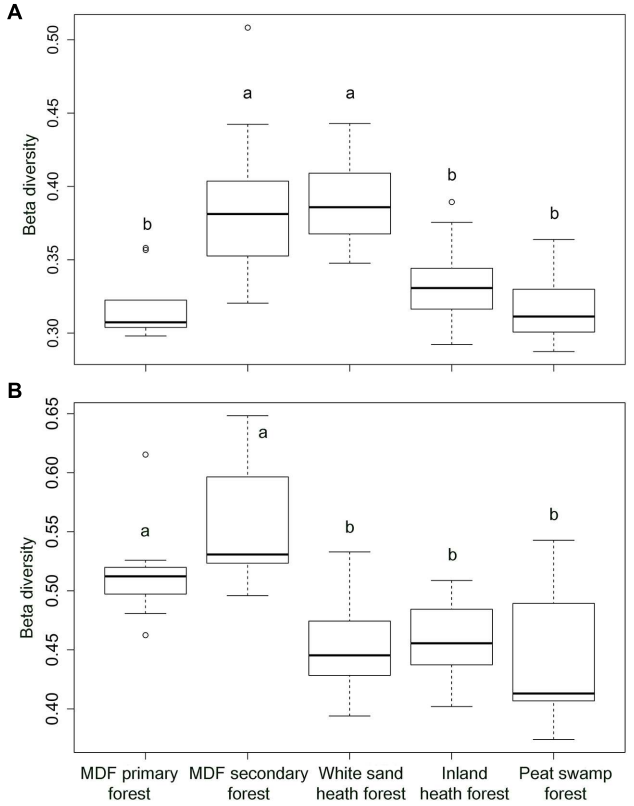

FIGURE 5 | The Whittaker beta-diversity of (A) bacterial community and (B) fungal community in five different forest types in Brunei, Borneo. Significant differences ($P < 0.05$) between forest types, when present, are indicated by different letters.

At the broad taxonomic level, the relative abundance of fungal taxa detected in this study is similar to the soils of other tropical regions, where Ascomycota and Basidiomycota are also the most predominant phyla (Kerfahi et al., 2014; McGuire et al., 2014). Compared to MDF primary forest, there is an increased proportion of Ascomycota in other forest types. Ascomycota are often found at higher abundance in stressful environments (De Beeck et al., 2015), and the communities here appears to reflect this pattern. However, the lower relative abundance of Basidiomycota in white sand heath forest, inland heath forest and peat swamp forest reflect the distinctive conditions in these forest types compared to MDF forests, as numerous Basidiomycota fungi tend to be slow-growing, late-successional fungi that are sensitive to physical and chemical perturbations (Frankland, 1998; Osono, 2007).

Are Distinctive Conditions Associated with Lower Fungal and Bacterial Diversity?

We expected to find lower alpha- and beta-diversity of bacteria and fungi in the more distinctive environments of the heath and peat swamp forests. This would be due to a combination of the low likelihood of lineages acquiring the evolutionary adaptations necessary to live in the conditions of low pH and water logged environments with limited oxygen supply. However, the observed diversity patterns did not follow these predictions. Alpha diversity of bacteria was higher in the white sand heath forest than in MDF primary forest, and similar to MDF secondary forest, while inland heath and peat swamp forest had almost similar level of alpha diversity to the MDF primary forest. When samples across all the forest types were compared in relation to soil parameters, pH emerged as overwhelmingly the strongest predictor of bacterial alpha diversity (**Figure 4A**). This result gives further confirmation of the generality of the pattern observed in other contexts around the world (Fierer and Jackson, 2006; Lauber et al., 2009; Tripathi et al., 2012), that bacterial alpha diversity increases toward neutral pH. Bacterial beta-diversity was highest in the white sand heath forest and in the MDF secondary forest – the same pattern as for bacterial alpha diversity. The other forest types had almost similar levels of bacterial beta diversity to one another. It appears that in this case tree species diversity has no bearing on the beta-diversity, perhaps reflecting the generally looser relationships between soil bacterial diversity and particular tree hosts (Millard and Singh, 2010).

Fungal alpha diversity was the same in all of the forest types except peat swamp forest. Thus, despite the apparently extreme conditions of two types of heath forests, fungal alpha diversity is no lower than in MDF primary or secondary forests. Only the peat swamp forest, perhaps because of waterlogged conditions, had lower fungal diversity. Beta diversity of fungi was, however, greater in the MDF primary and secondary forests than in the heath and peat swamp forests. This might be explicable in terms of the lower plant species diversity of these other non-terra firme forest types (Davies and Becker, 1996). Fungi often are involved in direct interactions with plants (Broeckling et al., 2008; Millard and Singh, 2010), and mycorrhizal fungi are specialized to grow under direct symbiotic relationships with plants (Gao et al., 2013). The greater beta diversity in the two terra firme forest types might then reflect the greater tree species diversity of these, with different samples able to reflect the range of host-tree-specific fungal communities that are present. An important role of the woody plant cover is also supported by the dominance of EcM fungi in MDF forests, EcM fungal groups are often dominant in Southeast Asian dipterocarp forests (Peay et al., 2010; Brearley, 2012; McGuire et al., 2014). Also importance might be a greater range of different saprotrophic fungal communities resulting from the input of different litter types from a more diverse assemblage of tree species.

Overall, this study confirmed our expectations that within the tropical rainforest, there is a strong degree of ecological differentiation in soil bacterial and fungal communities. However, the patterns in soil microbial diversity that we found amongst the various forest types in Brunei do not closely conform to our predictions that distinctive environments would show lower alpha- and beta-diversity of bacteria and fungi. There is need for further theoretical consideration to try to explain why the apparently distinctive and geologically 'ephemeral' environments of heath and peat swamp forests are about as diverse, or more

diverse, than terra firme MDF primary and secondary forests.

AUTHOR CONTRIBUTIONS

BT, JS, RS, and JA designed the study, BT, WS, JS, RS, and SJ completed fieldwork in Brunei, BT, WS, and SJ processed samples in the laboratory, BT and KD completed data processing and analysis, BT and JA produced the first draft of the manuscript, and all authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00376

REFERENCES

Abarenkov, K., Henrik Nilsson, R., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., et al. (2010). The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytol.* 186, 281–285. doi: 10.1111/j.1469-8137.2009.03160.x

Allen, S. E. (1989). *Chemical Analysis of Ecological Materials*. Oxford: Blackwell Scientific Publications.

Andriesse, J. (1988). *Nature and Management of Tropical Peat Soils*. Rome: Food and Agriculture Organization of the United Nations Soils Bulletin. 59, 165.

Araujo, J. F., de Castro, A. P., Costa, M. M., Togawa, R. C., Júnior, G. J. P., Quirino, B. F., et al. (2012). Characterization of soil bacterial assemblies in Brazilian savanna-like vegetation reveals acidobacteria dominance. *Microb. Ecol.* 64, 760–770.

Ashton, P. S. (1988). Dipterocarp biology as a window to the understanding of tropical forest structure. *Ann. Rev. Ecol. Syst.* 19, 347–370. doi: 10.1146/annurev.es.19.110188.002023

Becker, P. (1992). Seasonality of rainfall and drought in Brunei Darussalam. *Brunei Mus. J.* 7, 99–109.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57, 289–300.

Brearley, F. Q. (2012). Ectomycorrhizal associations of the Dipterocarpaceae. *Biotropica* 44, 637–648. doi: 10.1111/j.1744-7429.2012.00862.x

Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K., and Vivanco, J. M. (2008). Root exudates regulate soil fungal community composition and diversity. *Appl. Environ. Microbiol.* 74, 738–744. doi: 10.1128/AEM.02188-07

Bruenig, E., and Droste, H. (1995). "Structure, dynamics and management of rainforests on nutrient-deficient soils in Sarawak," in *Ecology, Conservation, and Management of Southeast Asian rainforests*, eds R. B. Primack and T. E. Lovejoy (London: Yale University Press), 41–53.

Brünig, E. F. (1974). *Ecological Studies in the Kerangas Forests of Sarawak and Brunei*. Kuching: Borneo Literature Bureau for Sarawak Forest Department.

Cannon, C. H., and Leighton, M. (2004). Tree species distributions across five habitats in a Bornean rain forest. *J. Veg. Sci.* 15, 257–266. doi: 10.1111/j.1654-1103.2004.tb02260.x

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Corlett, R. T. (2014). *The Ecology of Tropical East Asia*. Oxford: Oxford University Press.

David, A., and Sidup, S. (1996). *Brunei Metereological Services, Updated 2008, Climate of Brunei Darussalam*. Available at: http://www.bruneiweather.com.bn

Davies, S., and Becker, P. (1996). Floristic composition and stand structure of mixed dipterocarp and heath forests in Brunei Darussalam. *J. Trop. For. Sci.* 8, 542–569.

De Beeck, M. O., Ruytinx, J., Smits, M. M., Vangronsveld, J., Colpaert, J. V., and Rineau, F. (2015). Belowground fungal communities in pioneer Scots pine stands growing on heavy metal polluted and non-polluted soils. *Soil Biol. Biochem.* 86, 58–66. doi: 10.1016/j.soilbio.2015.03.007

Din, H., Metali, F., and Sukri, R. S. (2015). Tree diversity and community composition of the Tutong white sands, Brunei Darussalam: a rare tropical heath forest ecosystem. *Int. J. Ecol.* 2015:2015. doi: 10.1155/2015/807876

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 103, 626–631. doi: 10.1073/pnas.0507535103

Frankland, J. C. (1998). Fungal succession—unravelling the unpredictable. *Mycol. Res.* 102, 1–15. doi: 10.1017/S0953756297005364

Gao, C., Shi, N. N., Liu, Y. X., Peay, K. G., Zheng, Y., Ding, Q., et al. (2013). Host plant genus-level diversity is the best predictor of ectomycorrhizal fungal diversity in a Chinese subtropical forest. *Mol. Ecol.* 22, 3403–3414. doi: 10.1111/mec.12297

Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi: 10.1111/j.1365-294X.1993.tb00005.x

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., and Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–1579. doi: 10.1038/ismej.2011.41

Jesus, E. D., Marsh, T. L., Tiedje, J. M., and Moreira, F. M. D. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Jing, X., Sanders, N. J., Shi, Y., Chu, H., Classen, A. T., Zhao, K., et al. (2015). The links between ecosystem multifunctionality and above-and belowground biodiversity are mediated by climate. *Nat. Commun.* 6:8159. doi: 10.1038/ncomms9159

Jones, R. T., Robeson, M. S., Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J.* 3, 442–453. doi: 10.1038/ismej.2008.127

Kanokratana, P., Uengwetwanit, T., Rattanachomsri, U., Bunterngsook, B., Nimchua, T., Tangphatsornruang, S., et al. (2011). Insights into the phylogeny and metabolic potential of a primary tropical peat swamp forest microbial

community by metagenomic analysis. *Microb. Ecol.* 61, 518–528. doi: 10.1007/s00248-010-9766-7

Kerfahi, D., Tripathi, B. M., Lee, J., Edwards, D. P., and Adams, J. M. (2014). The impact of selective-logging and forest clearance for oil palm on fungal communities in Borneo. *PLoS ONE* 9:e111525. doi: 10.1371/journal.pone.0111525

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., et al. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* 62, 716–721. doi: 10.1099/ijs.0.038075-0

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi: 10.1128/AEM.00335-09

Lee-Cruz, L., Edwards, D. P., Tripathi, B. M., and Adams, J. M. (2013). Impact of logging and forest conversion to oil palm plantations on soil bacterial communities in Borneo. *Appl. Environ. Microbiol.* 79, 7290–7297. doi: 10.1128/AEM.02541-13

Leff, J. W., Jones, S. E., Prober, S. M., Barberán, A., Borer, E. T., Firn, J. L., et al. (2015). Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10967–10972. doi: 10.1073/pnas.1508382112

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *ISME J.* 5, 169–172. doi: 10.1038/ismej.2010.133

MacKinnon, K. (1996). *The Ecology of Kalimantan*. Oxford: Oxford University Press.

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., and Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31. doi: 10.1186/1471-2105-13-31

McGuire, K., D'Angelo, H., Brearley, F., Gedallovich, S., Babar, N., Yang, N., et al. (2014). Responses of soil fungi to logging and oil palm agriculture in Southeast Asian tropical forests. *Microb. Ecol.* 69, 733–747. doi: 10.1007/s00248-014-0468-4

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. doi: 10.1186/1471-2105-9-386

Millard, P., and Singh, B. (2010). Does grassland vegetation drive soil microbial diversity? *Nutr. Cycl. Agroecosys.* 88, 147–158. doi: 10.1007/s10705-009-9314-3

Miyashita, N. T., Iwanaga, H., Charles, S., Diway, B., Sabang, J., and Chong, L. (2013). Soil bacterial community structure in five tropical forests in Malaysia and one temperate forest in Japan revealed by pyrosequencing analyses of 16S rRNA gene sequence variation. *Genes Genet. Syst.* 88, 93–103. doi: 10.1266/ggs.88.93

Moran, J. A., Barker, M. G., Moran, A. J., Becker, P., and Ross, S. M. (2000). A comparison of the soil water, nutrient status, and litterfall characteristics of tropical heath and mixed-dipterocarp forest sites in Brunei1. *Biotropica* 32, 2–13. doi: 10.1646/0006-3606(2000)032[0002:ACOTSW]2.0.CO;2

Nacke, H., Fischer, C., Thürmer, A., Meinicke, P., and Daniel, R. (2014). Land use type significantly affects microbial gene transcription in soil. *Microb. Ecol.* 67, 919–930. doi: 10.1007/s00248-014-0377-6

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M., Oksanen, M., et al. (2007). *The Vegan Package. Community Ecology Package 2.0-7*.

Osono, T. (2007). Ecology of ligninolytic fungi associated with leaf litter decomposition. *Ecol. Res.* 22, 955–974. doi: 10.1007/s11284-007-0390-z

Pacchioni, R. G., Carvalho, F. M., Thompson, C. E., Faustino, A. L., Nicolini, F., Pereira, T. S., et al. (2014). Taxonomic and functional profiles of soil samples from Atlantic forest and Caatinga biomes in northeastern Brazil. *Microbiologyopen* 3, 299–315. doi: 10.1002/mbo3.169

Page, S., Rieley, J., and Wüst, R. (2006). Lowland tropical peatlands of Southeast Asia. *Dev Earth Surf. Proc.* 9, 145–172. doi: 10.1016/S0928-2025(06)09007-9

Peay, K. G., Kennedy, P. G., Davies, S. J., Tan, S., and Bruns, T. D. (2010). Potential link between plant and fungal distributions in a dipterocarp rainforest: community and phylogenetic structure of tropical ectomycorrhizal fungi across a plant and soil ecotone. *New Phytol.* 185, 529–542. doi: 10.1111/j.1469-8137.2009.03075.x

Prosser, J. I. (2012). Ecosystem processes and interactions in a morass of diversity. *FEMS Microbiol. Ecol.* 81, 507–519. doi: 10.1111/j.1574-6941.2012.01435.x

Pupin, B., and Nahas, E. (2014). Microbial populations and activities of mangrove, restinga and Atlantic forest soils from Cardoso Island, Brazil. *J. Appl. Microbiol.* 116, 851–864. doi: 10.1111/jam.12413

Rodrigues, J. L., Pellizari, V. H., Mueller, R., Baek, K., Jesus Eda, C., Paula, F. S., et al. (2013). Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 110, 988–993. doi: 10.1073/pnas.1220608110

Russo, S. E., Legge, R., Weber, K. A., Brodie, E. L., Goldfarb, K. C., Benson, A. K., et al. (2012). Bacterial community structure of contrasting soils underlying Bornean rain forests: inferences from microarray and next-generation sequencing methods. *Soil Biol. Biochem.* 55, 48–59. doi: 10.1016/j.soilbio.2012.05.021

Satrio, A. E., Gandaseca, S., Ahmed, O. H., and Nik Muhamad, A. (2009). Effect of precipitation fluctuation on soil carbon storage of a tropical peat swamp forest. *Am. J. Appl. Sci.* 6, 1484–1488. doi: 10.3844/ajassp.2009.1484.1488

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Slik, J. W. F., Poulsen, A., Ashton, P., Cannon, C., Eichhorn, K., Kartawinata, K., et al. (2003). A floristic analysis of the lowland dipterocarp forests of Borneo. *J. Biogeogr.* 30, 1517–1531. doi: 10.1046/j.1365-2699.2003.00967.x

Slik, J. W. F., Raes, N., Aiba, S. I., Brearley, F. Q., Cannon, C. H., Meijaard, E., et al. (2009). Environmental correlates for tropical tree diversity and distribution patterns in Borneo. *Divers. Distrib.* 15, 523–532. doi: 10.1111/j.1472-4642.2009.00557.x

Sukri, R. S., Wahab, R. A., Salim, K. A., and Burslem, D. F. (2012). Habitat associations and community structure of dipterocarps in response to environment and soil conditions in Brunei Darussalam, northwest Borneo. *Biotropica* 44, 595–605. doi: 10.1111/j.1744-7429.2011.00837.x

Tedersoo, L., May, T. W., and Smith, M. E. (2010). Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* 20, 217–263. doi: 10.1007/s00572-009-0274-x

Tripathi, B. M., Kim, M., Lai-Hoe, A., Shukor, N. A. A., Rahim, R. A., Go, R., et al. (2013). pH dominates variation in tropical soil archaeal diversity and community structure. *FEMS Microbiol. Ecol.* 86, 303–311. doi: 10.1111/1574-6941.12163

Tripathi, B. M., Kim, M., Singh, D., Lee-Cruz, L., Lai-Hoe, A., Ainuddin, A., et al. (2012). Tropical soil bacterial communities in Malaysia: pH dominates in the equatorial tropics too. *Microb. Ecol.* 64, 474–484. doi: 10.1007/s00248-012-0028-8

Tripathi, B. M., Lee-Cruz, L., Kim, M., Singh, D., Go, R., Shukor, N. A., et al. (2014). Spatial scaling effects on soil bacterial communities in Malaysian tropical forests. *Microb. Ecol.* 68, 247–258. doi: 10.1007/s00248-014-0404-7

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press), 315–322.

Whitmore, T. (1984). *Tropical Rain Forests of the Far East*, 2nd Edn. Oxford: Oxford University Press.

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High resolution depth distribution of *Bacteria*, *Archaea*, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy

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The communities and abundances of methanotrophs and methanogens, along with the oxygen, methane, and total organic carbon (TOC) concentrations, were investigated along a depth gradient in a flooded rice paddy. Broad patterns in vertical profiles of oxygen, methane, TOC, and microbial abundances were similar in the bulk and rhizosphere soils, though methane and TOC concentrations and 16S rRNA gene copies were clearly higher in the rhizosphere soil than in the bulk soil. Oxygen concentrations decreased sharply to below detection limits at 8 mm depth. Pyrosequencing of 16S rRNA genes showed that bacterial and archaeal communities varied according to the oxic, oxic-anoxic, and anoxic zones, indicating that oxygen is a determining factor for the distribution of bacterial and archaeal communities. Aerobic methanotrophs were maximally observed near the oxic-anoxic interface, while methane, TOC, and methanogens were highest in the rhizosphere soil at 30–200 mm depth, suggesting that methane is produced mainly from organic carbon derived from rice plants and is metabolized aerobically. The relative abundances of type I methanotrophs such as *Methylococcus*, *Methylomonas*, and *Methylocaldum* decreased more drastically than those of type II methanotrophs (such as *Methylocystis* and *Methylosinus*) with increasing depth. *Methanosaeta* and *Methanoregula* were predominant methanogens at all depths, and the relative abundances of *Methanosaeta*, *Methanoregula*, and *Methanosphaerula*, and GOM_Arc_I increased with increasing depth. Based on contrasts between absolute abundances of methanogens and methanotrophs at depths sampled across rhizosphere and bulk soils (especially millimeter-scale slices at the surface), we have identified populations of methanogens (*Methanosaeta*, *Methanoregula*, *Methanocella*, *Methanobacterium*, and *Methanosphaerula*), and methanotrophs (*Methylosarcina, Methylococcus, Methylosinus,* and unclassified *Methylocystaceae*) that are likely physiologically active *in situ*.

Keywords: methanogen, methanotroph, flooded rice paddy, depth distribution, methane, bulk and rhizosphere soil

Introduction

Methane (CH_4) is the second most important greenhouse gas in the atmosphere, after carbon dioxide. It is produced by methanogens in the biosphere, but a large part of the released methane is metabolized by methanotrophs before it reaches the atmosphere (Bridgham et al., 2013). Therefore, methane emission from the biosphere into the atmosphere is mainly determined by methanogenic and methanotrophic activities, and numerous prior studies have focused on methanogens and methanotrophs to gain a better understanding of methane metabolism in the biospheric carbon cycles (Semrau et al., 2010; Bridgham et al., 2013; Costa and Leigh, 2014). Among methanogenic groups, members of the orders *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, and *Methanocellales* have been identified from rice paddies (Sakai et al., 2009; Ma et al., 2012; Lee et al., 2014; Liu et al., 2015). Although anaerobic methanotrophs such as the anaerobic methanotrophic archaea (ANME), "*Candidatus* Methylomirabilis oxyfera," and "*Candidatus* Methanoperedens nitroreducens" have recently been reported as putatively important players in methane oxidation (Ettwig et al., 2010; Biddle et al., 2012; Haroon et al., 2013; Shen et al., 2014), it has been suggested that methane is metabolized mainly by aerobic methanotrophs in rice paddies (Groot et al., 2003; Ma et al., 2010, 2013; Lee et al., 2014). Based on their physiological characteristics and phylogeny, the aerobic methanotrophs are generally divided into two groups, types I and II, corresponding to the family *Methylococcaceae* (*Gammaproteobacteria*) and the families *Methylocystaceae* and *Beijerinckiaceae* (*Alphaproteobacteria*), respectively (Semrau et al., 2010; Lüke and Frenzel, 2011). In addition, atypical methanotrophs belonging to the family *Methylacidiphilaceae* of the phylum *Verrucomicrobia* were reported to be aerobic (Op den Camp et al., 2009).

Rice, one of the most important crop plants worldwide with a total cultivation area of 155 million hectares, is traditionally grown under flooded or wet conditions during most of the cultivation period (Ma et al., 2010). Because the waterlogged soil of rice paddies provides ideal conditions for methanogenesis, rice field is considered an important anthropogenic methane source accounting for 5–19% of the global methane emission to the atmosphere (IPCC, 2007; Ma et al., 2010). Methane emission from rice paddies is the result of complicated processes, including hydrolysis and fermentation of organic matter, methanogenesis, and methane transport and oxidation, which involve complex consortia of hydrolytic, fermenting, methanogenic, and methanotrophic microorganisms (Liesack et al., 2000; Conrad, 2002, 2007). Previous studies have shown that different methanotrophic and methanogenic taxa display varying sensitivities and responses to oxygen concentrations (Gilbert and Frenzel, 1998; Yuan et al., 2009, 2011; Krause et al., 2010; Ma et al., 2012; Reim et al., 2012). Indeed, the abundance and distribution of methanogenic and methanotrophic communities both respond to and create the physical and chemical gradients that occur with depth in flooded rice paddy soils (Lüdemann et al., 2000; Noll et al., 2005; Krause et al., 2010; Reim et al., 2012; Ma et al., 2013).

Because it is well known that organic matter derived from detritus and root exudates of rice plants is an important driver of methane production and that oxygen influx occurs through the aerenchyma of rice roots, the abundance and structure of the methanogenic and methanotrophic communities along a depth gradient are expected to differ between the bulk and rhizosphere soils of planted and unplanted rice paddies. However, previous studies of microbial communities in rice paddies have largely relied upon terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes (Lüdemann et al., 2000; Noll et al., 2005; Reim et al., 2012). Although T-RFLP has been successfully used by some research groups to analyze methanogenic and methanotrophic communities (Ma and Lu, 2011; Ma et al., 2013), T-RPLP can deliver only limited insights into complex communities. Pyrosequencing is a more powerful technique to explore the composition of microbial communities in natural habitats (Roesch et al., 2007), and recently pyrosequencing has been used to analyze methanogen and methanotroph communities in rice paddies (Lee et al., 2014; Lüke et al., 2014; Breidenbach and Conrad, 2015). However, it has not yet been applied to samples gathered at millimeter resolution.

In this study, we combined the millimeter-scale sampling with pyrosequencing-based community characterization of communities, especially methanotrophs and methanogens, in bulk and rhizosphere soils of a planted rice paddy. Furthermore, we determined the oxygen, methane, and total organic carbon (TOC) concentrations in the corresponding depths. Thus, the findings reported here expand the current knowledge and understanding of methane production, oxidation, and transport in rice paddies.

Materials and Methods

Rice Paddy Description and Soil Sampling along a Depth Gradient

Soil cores were samples from a rice paddy located in Sacheon, South Korea (35°10′90″N, 128°11′84″E). The rice paddy has a silt loam soil texture (20% clay, 55% silt, 25% sand) and has been tilled once a year for the last several decades. All farming practices, including tillage, transplanting of the Korean rice cultivar Dongjinbyeo (*Oryza sativa*, Japonica type), water irrigation, and chemical fertilization, were performed according to the same procedures described previously (Lee et al., 2014). Soil core sampling in the flooded rice paddy was performed on September 3, 2013, approximately 90 days after rice-transplanting (flowering and heading stages of rice growth), as methane emission reaches a maximal level during this phase of the rice cultivation period (Lee et al., 2014). Prior to the soil core sampling, oxygen concentrations along a depth gradient in the bulk and rhizosphere soils of the rice paddy were measured *in situ* using the Fibox 3 LCD trace system with the Pst3 sensor (PreSens GmbH, Rogensburg, Germany). Soil cores (3-cm diameter and 45-cm length) were collected from the bulk soil (at the mid-diagonal point separating individual plants placed at 30 × 15 cm spacings) and from the rhizosphere soil (∼3 cm from the base of rice plants) within the flooded rice paddy (Supplementary

Figure S1), using polyvinyl chloride soil core samplers sealed with rubber stoppers.

The sampled tubes were immediately frozen in a dry ice/ethanol bath, and then stored at −80°C until further analysis.

For the analysis of the microbial communities and methane concentrations along a depth gradient, 1.0-mm-thick sections were cut from the surface of the frozen soil cores to a 10-mm depth using a microtome cryostat (HM 505E, Microm, Germany) as described previously (Reim et al., 2012). In addition, soil samples corresponding to 30, 60, 100, 200, 300, and 400 mm depth were cut from the frozen soil cores using the microtome cryostat. Half of each frozen soil subsample was transferred into a 10-ml serum bottle, which was immediately sealed with rubber stoppers for the analysis of the methane concentration. The remaining half of soil subsamples were stored at −80°C until the analysis of the microbial community structure and abundance.

Analysis of Methane and TOC Concentrations

To measure methane concentrations along a depth gradient in the rice paddy, the 10-ml serum bottles containing the soil samples were warmed to 25°C and shaken for 1 min after injection of 1 ml of distilled water (DW). Headspace gas samples (0.5 ml) were taken from the serum bottles using a gas-tight syringe (Hamilton, USA) and methane concentrations were measured with a 6890N gas chromatograph (GC, Agilent Technologies, USA) equipped with a flame-ionization detector and an HP-5 capillary column (0.32 mm × 30 m, 0.25 μm film thickness, J & W Scientific, USA) as described previously (Herman and Roberts, 2006). The methane concentrations in the rice paddy soil were calculated on a fresh weight basis by measuring the weights of the soil subsamples used for methane analysis. The measurements were performed in triplicate.

To measure TOC concentrations along a depth gradient, seven soil samples corresponding to 0–10, 25–35, 55–65, 95–105, 195–205, 295–305, and 395–405 mm depths in the bulk and rhizosphere soils of the rice paddy were collected from the frozen soil cores using a small handsaw. Twenty-five milliliters of DW were added to 5 g of the moist soil samples and the mixtures were agitated for 60 min using a shaker at 250 rpm. The mixtures were centrifuged at 8000 rpm (∼7200 g) for 10 min and the supernatants were filtered using 0.45-μm pore-size filters. The TOC concentrations of the filtrates were measured using a TOC analyzer (TOC-VCPH, Shimadzu, Japan). The TOC concentrations in the rice paddy were calculated on a dry weight basis by measuring the dry weight of the precipitates after centrifugation. The measurements were performed in triplicate.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

To estimate the bacterial and archaeal abundances along a depth gradient in the rice paddy, qPCR was performed as described previously with some modifications (Lee et al., 2012, 2014). Briefly, genomic DNA was extracted from the remaining half of frozen soil subsamples using a Fast DNA spin kit (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. Two qPCR primer sets, bac1114F (5′-CGG CAA CGA GCG CAA CCC-3′)/bac1275R (5′-CCA TTG TAG CAC GTG TGT AGC C-3′) and arch349F (5′-GYG CAS CAG KCG MGA AW-3′)/arch806R (5′-GGA CTA CVS GGG TATC TAA T-3′), were used to amplify the 16S rRNA genes of *Bacteria* and *Archaea*, respectively (Takai and Horikoshi, 2000; Denman and McSweeney, 2006). The qPCR amplifications were performed in triplicate and gene copy numbers were estimated by using two standard curves generated from pCR2.1 vectors (Invitrogen, USA) carrying bacterial (*Chloroflexi*) and archaeal (a methanogenic archaeon) 16S rRNA genes as described previously (Jung et al., 2011; Lee et al., 2012). Bacterial and archaeal 16S rRNA gene copy numbers in the rice paddy were calculated on a fresh weight basis by measuring the weight of the soil samples used for DNA extraction.

Analysis of the Bacterial and Archaeal Community Structure using Pyrosequencing

For the analysis of bacterial and archaeal community structures along a depth gradient in the rice paddy, composite genomic DNA samples were prepared by mixing equal amounts of the genomic DNA extracted from the same depths of three different cores. The hypervariable regions of bacterial and archaeal 16S rRNA genes were amplified using primer sets Bac9F/Bac541R and Arc344F/Arc927R with unique 7–11 mer barcode sequences, respectively (Supplementary Table S1). The PCR products were pooled for pyrosequencing using a 454 GS-FLX Titanium system (Roche, Mannheim, Germany) at Macrogen (Seoul, Korea) as described previously (Lee et al., 2014).

The pyrosequencing data were processed using RDPipeline (http://pyro.cme.msu.edu; Cole et al., 2009). The pyrosequencing reads were sorted to the specific samples based on their unique barcodes, after which the barcode and primer sequences were trimmed. Reads with more than two undetermined nucleotides and/or read lengths shorter than 300 bp were excluded from subsequent analysis, and potential chimeric reads were discarded by using the UCHIME chimera slayer (Edgar et al., 2011) in RDPipeline. To compare the microbial diversity among the soil samples, the bacterial and archaeal read numbers of each sample were normalized to those of the sample with the smallest number of reads by random removal of sequencing reads using the sub.sample command of the Mothur program (Schloss et al., 2009). Operational taxonomic units (OTUs), Shannon–Weaver (Shannon and Weaver, 1963), and Chao1 biodiversity (Chao, 1987) indices and evenness for the normalized sequencing reads were computed with RDPipeline at a 97% identity cutoff value.

The bacterial and archaeal communities along a depth gradient in the bulk and rhizosphere soils of the rice paddy were compared using the UniFrac analysis (Lozupone and Knight, 2005). Representative sequences were selected by aligning the normalized reads and clustering them at a 97% nucleotide identity cutoff in RDPipeline, and singletons were removed as described by Zhou et al. (2011). The representative sequences without singletons were aligned in RDPipeline and neighbor-joining (NJ) trees were constructed based on the Kimura two-parameter model using the PHYLIP software (ver. 3.695) (Felsenstein, 2002). The NEXUS files of the NJ trees were used as input files for weighted hierarchical clustering and principal coordinate analysis (PCoA).

Taxonomic classification was performed for all high-quality sequences of *Bacteria* and *Archaea* at the phylum, class, and genus levels based on the SILVA database (v.102), using the nearest-neighbor method within the mothur program (Pruesse et al., 2007). The absolute abundances of methanotrophic and methanogenic groups along a depth gradient in the bulk and rhizosphere soils of the rice paddy were estimated by multiplying the relative abundances of methanotrophic and methanogenic groups that were classified at the genus level and the gene copy numbers of bacterial and archaeal 16S rRNA genes that were obtained by the qPCR analysis.

Nucleotide Sequence Accession Number

The pyrosequencing data of the bacterial and archaeal 16S rRNA genes are publicly available in the NCBI Short Read Archive (SRA) under accession no. SRP052852 (NCBI BioProject PRJNA273696).

Results

Vertical Profiles of Oxygen, Methane, and TOC Concentrations

The concentration profiles of oxygen, methane, and TOC along a depth gradient in the flooded rice paddy are shown in **Figure 1**. The oxygen concentration in the surface floodwater of the paddy soil was approximately 8.8 ppm, but the concentration decreased very sharply to below the detection limit at 8-mm (**Figure 1A**). This result suggested that the oxic-anoxic interface representing the penetration limit of oxygen from the soil surface was located at the depth range of approximately 6–10-mm, which was slightly different from previously reported results in which the oxic-anoxic interfaces were located at approximately 2-mm depth (Revsbech et al., 1999; Reim et al., 2012). This difference might be explained by the high oxygen concentration in the floodwater in this study. No clear difference was observed in the vertical oxygen profiles of the bulk and rhizosphere soils. The methane concentrations were extremely low in the aerobic surface soil (<0.1 μg/g-soil) (**Figure 1A**). Simultaneous depletion of oxygen and methane in the 4–10 mm oxic-anoxic interface zone is characteristic of aerobic methanotrophy. Below this interface zone, where aerobic methanotrophy certainly occurred *in situ*, methane concentration increased steadily with depth to maximum levels of 2.7 and 3.3 μg/g-soil at 60–100-mm in the bulk and rhizosphere soils, respectively; methane then decreased to approximately 0.4 μg/g-soil at the 400-mm depth in both soils (**Figure 1A**). Interestingly, the methane concentration at a depth of 9 mm in the rhizosphere soil was nearly double that at the same depth in the bulk soil. The TOC concentrations increased to maximum levels of 8.7 and 14.7 mg/g-soil at approximately 100-mm depth in the bulk and rhizosphere soils, respectively (**Figure 1B**). The vertical profiles of TOC corresponded well with those of methane concentrations. As expected, both the methane and TOC concentrations were generally higher in the rhizosphere than in the bulk soil of the rice paddy.

Abundances of *Bacteria* and *Archaea* along a Depth Gradient

To estimate the bacterial and archaeal abundances along a depth gradient in the bulk and rhizosphere soils of the rice paddy, a qPCR approach targeting the 16S rRNA genes was used (**Figure 2**). The total numbers of 16S rRNA gene copies of

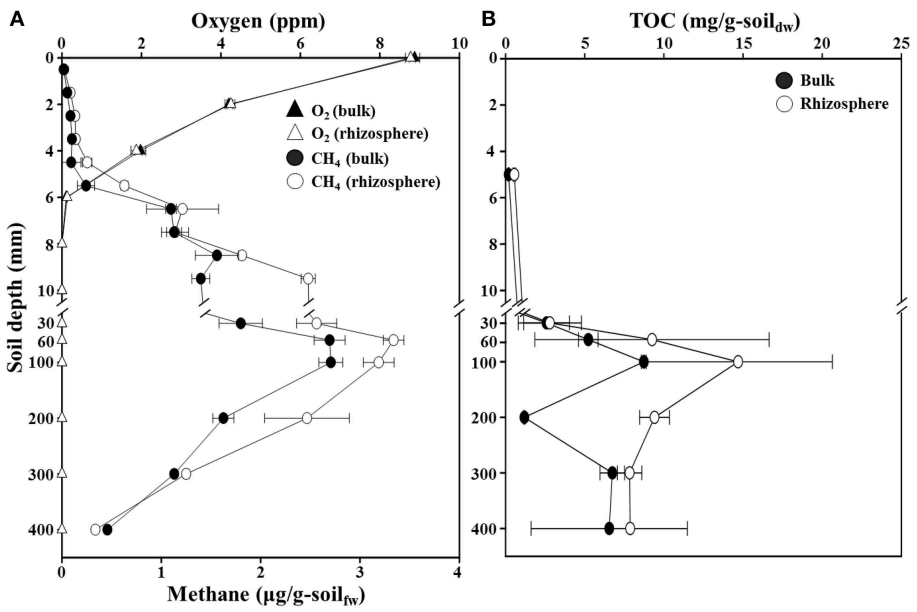

FIGURE 1 | Profiles of oxygen, methane (A), and total organic carbon (TOC) (B) concentrations along a depth gradient in the bulk and rhizosphere soil of the flooded rice paddy. The measurements were performed in triplicate. Errors bars indicate standard deviations ($n = 3$). g-soil$_{fw}$, gram-soil fresh weight; g-soil$_{dw}$, gram-soil dry weight.

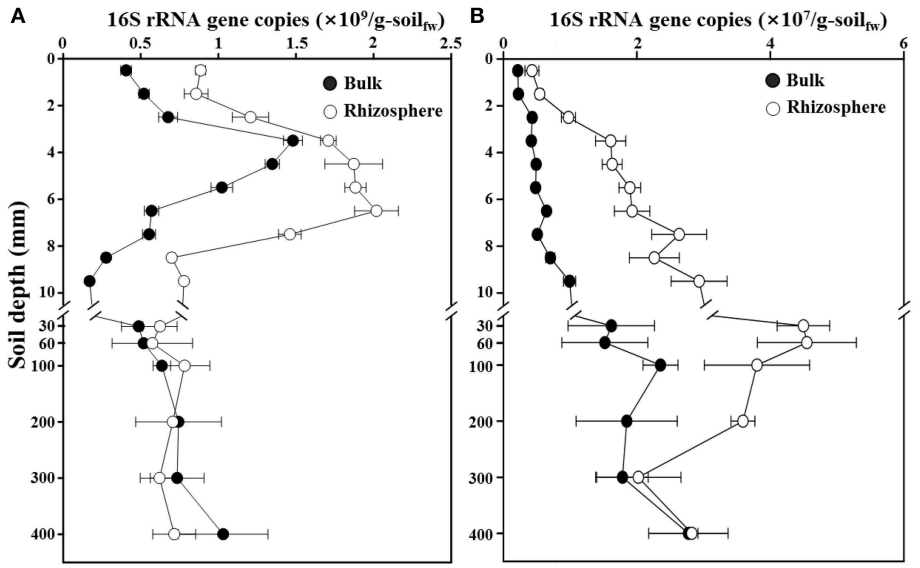

FIGURE 2 | Profiles of the 16S rRNA gene copies for total *Bacteria* (A) and *Archaea* (B) along a depth gradient in the bulk and rhizosphere soil of the flooded rice paddy. The measurements were performed in triplicate. Errors bars indicate standard deviations ($n = 3$). g-soil$_{fw}$, gram-soil fresh weight.

Bacteria were approximately 4.1×10^8 and 8.9×10^8 copies/g-soil at the surface of the bulk and rhizosphere soils, respectively. The copy numbers increased sharply to a maximum of 1.5×10^9 copies/g-soil at 3–4-mm depth and 2.0×10^9 copies/g-soil at 7–8-mm depth in the bulk and rhizosphere soils, respectively, and then rapidly decreased to numbers similar to those at the surface, at 10-mm depth (**Figure 2A**). These results are consistent with the hypothesis that organic carbon, including methane, may be supporting the *in situ* growth (hence high populations) of *Bacteria* at the oxic-anoxic interface of the rice paddy. Below 10-mm depth, which corresponds to anaerobic conditions, the bacterial 16S rRNA gene copy numbers remained relatively constant at approximately 7.0×10^8 copies/g-soil in both the bulk and rhizosphere soils. The 16S rRNA gene copy numbers of *Archaea* were approximately 2.2×10^6 and 4.3×10^6 copies/g-soil at the surface of the bulk and rhizosphere soils, respectively (**Figure 2B**). The copy numbers increased to a maximum of 2.4×10^7 and 4.5×10^7 copies/g-soil at 60–100-mm depth in the bulk and rhizosphere soils, respectively, and then gradually decreased below 100-mm depth with increasing depth in the rice paddy. The 16S rRNA gene copy numbers of *Bacteria* were higher in the rhizosphere than in the bulk soil at 0–30-mm depth, while those of *Archaea* were higher in the rhizosphere than in the bulk soil at all sampled depths.

Bacterial and Archaeal Community Composition along a Depth Gradient

To investigate the phylogenetic structure of the bacterial and archaeal communities along a depth gradient in the bulk and rhizosphere soils of the rice paddy, a parallel pyrosequencing approach was applied. After the removal of low-quality and putative chimeric reads, and trimming of the barcoded PCR primers, a total of 163,731 and 116,773 high-quality reads with corresponding average read lengths of 474 and 495 bases were obtained for the bacterial and archaeal communities, respectively (Supplementary Table S2). Because the read numbers influence the statistical diversity indices, especially the Chao1 and Shannon–Weaver indices, the numbers of the bacterial and archaeal reads in each sample were normalized to 3031 and 2122 reads, respectively, and the statistical diversity indices were calculated based on the normalized samples (Table S2). The Chao1 and Shannon–Weaver indices indicated that the bacterial diversities were relatively constant over the first 10-mm in both the bulk and rhizosphere soils, but they decreased below the 30-mm depth (**Figure 3A**), suggesting that the oxygen concentration is an important factor for bacterial diversity in the rice paddy. The archaeal diversities decreased steadily in both soils between the surface and 400-mm depth with increasing depth (**Figure 3B**). However, no obvious differences in the bacterial and archaeal diversities along a depth gradient were observed between the bulk and rhizosphere soils of the rice paddy.

The composition of the bacterial and archaeal communities along a depth gradient in the bulk and rhizosphere soils of the rice paddy was statistically compared using weighted UniFrac clustering and PCoA. Because Zhou et al. (2011) demonstrated that singletons can cause data distortion, these were removed from the normalized data before clustering and PCoA (**Figure 4**). The sequences of *Bacteria* were clustered three groups according to depths of 0–6, 6–10, and 30–400 mm (**Figure 4A**), corresponding with the oxic zone, the oxic-anoxic interface, and the anoxic zone, respectively, based on the vertical oxygen profiles as shown in **Figure 1A**. The sequences of *Archaea* were clustered into three groups according to depth; however, the clusters were less tightly related to the vertical oxygen profiles than that of the bacterial community data, especially in the oxic-anoxic interface of the rhizosphere soil

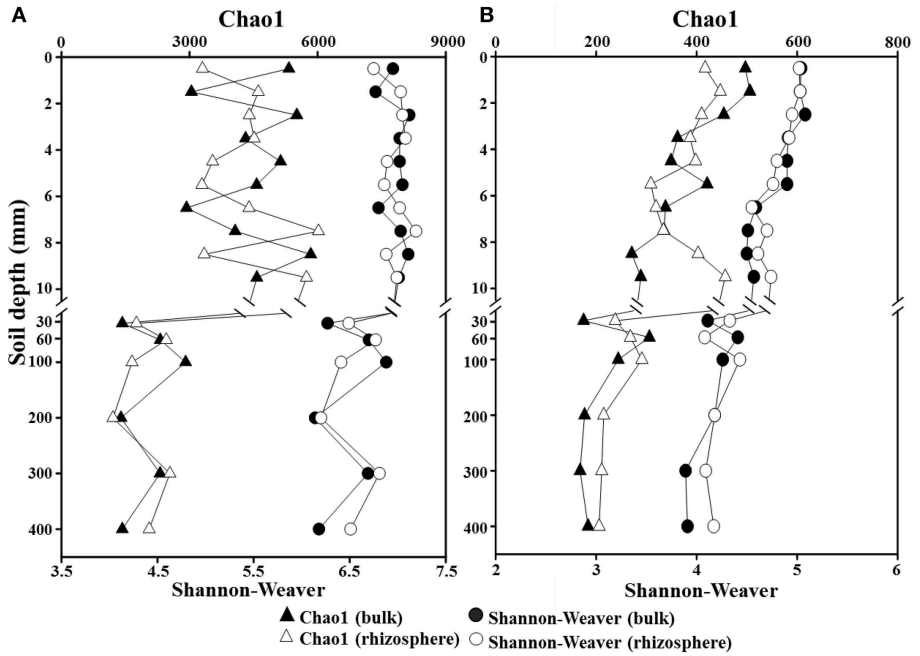

FIGURE 3 | Profiles of Chao1 and Shannon–Weaver indices for total *Bacteria* (A) and *Archaea* (B) along a depth gradient in the bulk and rhizosphere soils of the flooded rice paddy. Both indices were computed using the normalized pyrosequencing read data at a 97% identity cutoff value.

(**Figure 4B**). The PCoA results confirmed that the bacterial and archaeal communities could be divided into three groups corresponding with the vertical oxygen profiles (**Figures 4C,D**). However, the bacterial and archaeal communities were not clearly differentiated between the bulk and rhizosphere soil of the rice paddy. Taken together, these results showed that the composition of the bacterial and archaeal communities of the rice paddy differed along a depth gradient and suggested that the oxygen concentration might be a determining factor in this.

Next, the high-quality bacterial and archaeal sequences were taxonomically classified at the phylum and class levels, respectively, using the SILVA reference database (**Figure 5**). The classification showed that the bacterial and archaeal communities were not distinctly differentiated between the bulk and rhizosphere soils of the rice paddy. Predominant *Bacteria* belonged to the phyla, *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, and *Firmicutes* at all depths in both the bulk and rhizosphere soils (**Figures 5A,B**), although their relative abundances were slightly different along a depth gradient. The relative abundances of *Cyanobacteria* and *Bacteroidetes* decreased, while those of *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* increased with depth in both soils. The taxonomic classification of the archaeal sequences showed that methanogens, including *Methanomicrobia*, *Methanobacteria*, and *Methanococci*, which belong to the phylum *Euryarchaeota* were predominant at all depths in both the bulk and rhizosphere soils (**Figures 5C,D**). The relative abundance of the methanogens, especially *Methanomicrobia*, increased with depth, while non-methanogenic *Halobacteria*, which were predominant at the surface layer, sharply decreased with depth and formed a minority at the deep soil layer. These results suggested that *Halobacteria* in the rice paddy are aerobic, which was consistent with previously reported results that archaeal isolates belonging to *Halobacteria* are aerobic (Wainø et al., 2000; Shimane et al., 2011). In addition, Soil_Crenarchaeotic_Group (SCG) and Group_C3 belonging to the phylum *Crenarchaeota* were detected at high relative abundance and their relative abundances gradually increased with depth in both the bulk and rhizosphere soils (**Figures 5C,D**). The changes in the bacterial and archaeal communities were more pronounced at the surface layer (∼10 mm) associated with a sharp oxygen gradient, suggesting that oxygen might be a determining factor for the taxonomic composition of the microbial communities.

We sought deeper insights into the paddy soil microbial communities by moving from phylum- and class-level analyses (**Figure 5**) to the more rarified genus level. Here we focused on bacterial and archaeal sequences specifically involved in methane metabolic processes—methanogens and aerobic methanotrophs along the depth gradient in the rice paddy soil (**Figures 6**, **7**). Only 0.25–3.27% of the bacterial sequences were assigned as methanotrophs, while 37.3–88.1% of the archaeal sequences were recognized as methanogens (**Figures 6A–D**, respectively); these proportions of methanotrophs and methanogens were consistent with previously reported results (Lee et al., 2014). The relative abundances of the methanotrophs in the bulk soil increased in the surface layer and their maximum abundance was observed at 3–4-mm depth, but they gradually decreased with depth (**Figure 6A**). Whereas, the relative abundances of the methanotrophs in the rhizosphere soil showed a maximum level at the surface layer (1–3 mm) and then gradually

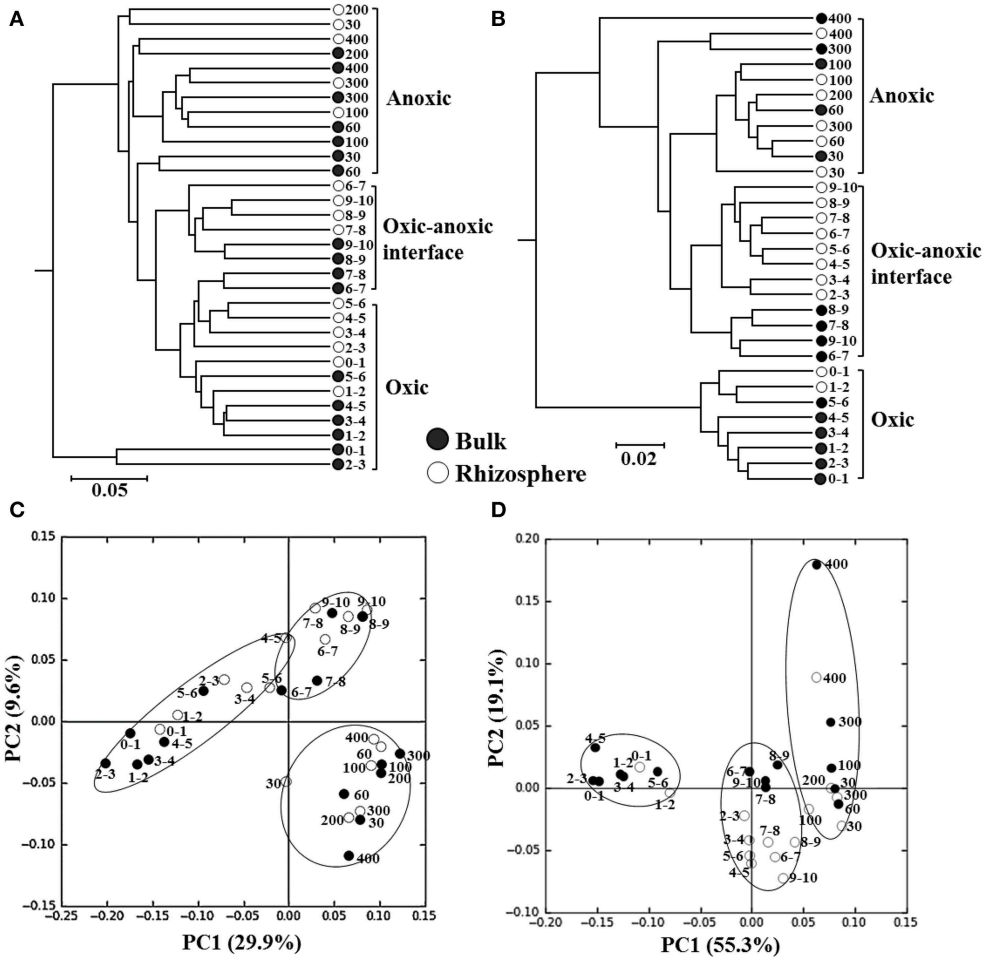

FIGURE 4 | The weighted UniFrac clustering (A,B) and PCoA (C,D) showing the relationships of bacterial (A,C) and archaeal (B,D) communities along a depth gradient in the bulk and rhizosphere soils of the flooded rice paddy. Numbers beside the symbols represent the depth (mm) from the surface. The scale bars in the trees represent the weighted UniFrac distances.

decreased with depth (**Figure 6B**). Members of *Methylosinus*, *Methylocystis*, *Methylococcus*, *Methylomonas*, and *Methylosarcina* were detected as dominant methanotrophs residing in both the bulk and rhizosphere soils, and their relative abundances differed along a depth gradient. The relative abundance of type II methanotrophs was nearly constant or decreased slowly with depth in both the bulk and rhizosphere soils, while that of type I methanotrophs, in particular the type Ib *Methylococcus*, decreased more sharply with depth. In contrast to the methanotrophs, the relative abundances of the methanogens increased with depth, and a pronounced increase was observed around the oxic-anoxic interface (**Figures 6C,D**). *Methanosaeta*, *Methanoregula*, *Methanocella*, *Methanosarcina*, *Methanobacterium*, *Methanosphaerula*, and GOM_Arc_I (Gulf of Mexico_*Archaea*_I) were the dominant methanogens in both soils, and their relative abundances differed along a depth gradient. The relative abundances of the genera *Methanosaeta*, *Methanoregula*, *Methanosphaerula*, and GOM_Arc_I, which belong to the class *Methanomicrobia*, increased with depth. In particular, GOM_Arc_I was one of the predominant members at the deep soil layer (400 mm). Species of the ANME group were hardly detected at all depths of the rice paddy, which was consistent with previously reported results (Lee et al., 2014).

Trends in the rice paddy methane profile in **Figure 1A** (drastic methane depletion at the 4–10 mm depths in both bulk and rhizosphere soils and a methane concentration at 9 mm in the rhizosphere soil nearly double that that of the bulk soil) were not readily interpretable based on the relative abundances of methanotrophs and methanogens shown in **Figure 6**. With the goal of identifying populations potentially involved in *in situ* methane production and consumption, we next estimated the absolute abundances (16S rRNA gene copies) of methanotrophic and methanogenic populations. To accomplish this, we multiplied the relative abundances of each group of populations with their 16S rRNA gene copy numbers (**Figure 7**). Absolute abundances of total methanotrophs and methanogens were $\sim 10^7$ 16S rRNA gene copies/g-soil in both the bulk and rhizosphere soils. This result was in agreement

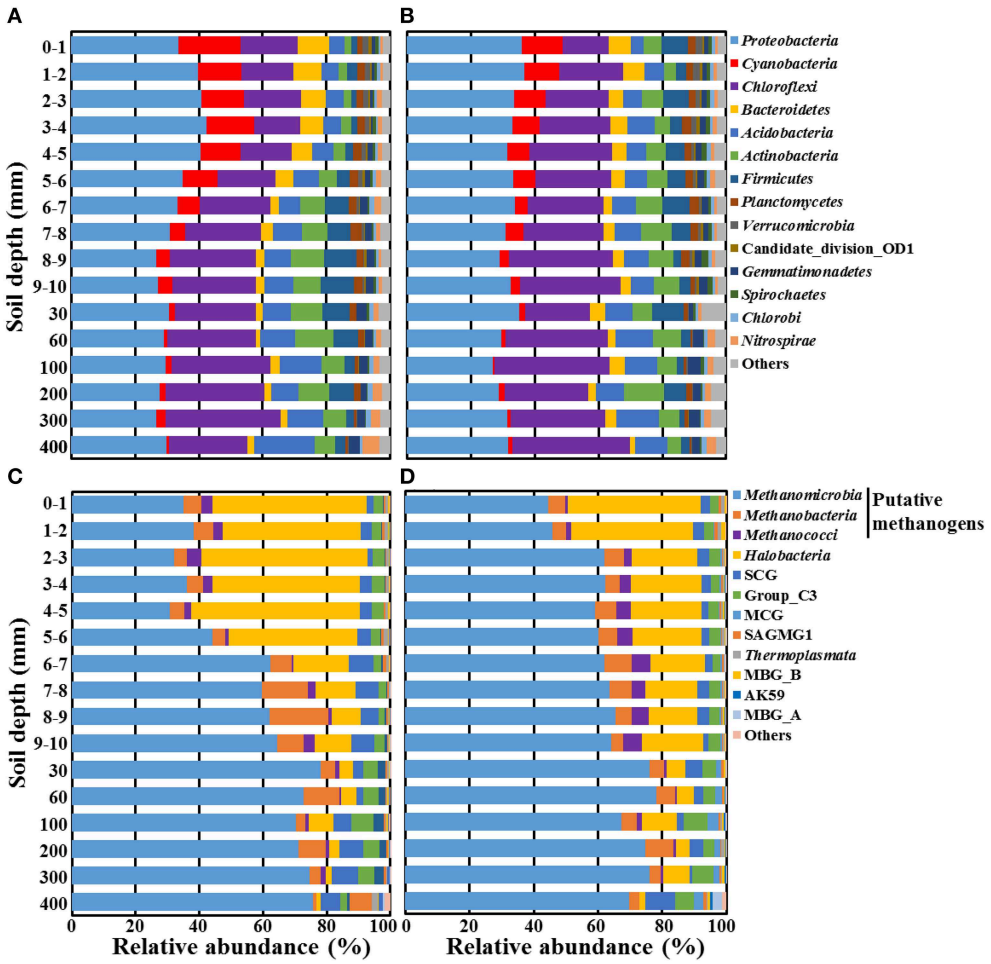

FIGURE 5 | Taxonomic compositions of bacterial (A,B) and archaeal (C,D) communities along a depth gradient in the bulk (A,C) and rhizosphere (B,D) soils of the rice paddy. The bacterial and archaeal 16S rRNA gene sequences were classified at the phylum and class levels, respectively, by using the mothur program based on the SILVA database. "Others" represents taxa that comprised <1 and 0.1% of the total reads in all of the bacterial and archaeal samples, respectively. SCG, Soil_Crenarchaeotic_Group; MCG, Miscellaneous_Crenarchaeotic_Group; SAGMG1, South_Africa_Gold_Mine_Goup_1; MBG, Marine_Benthic_Group.

with previously reported results, in which the abundances of methanotrophs and methanogens were analyzed by using qPCR of the methyl-coenzyme M reductase (*mcrA*) and particulate methane monooxygenase (*pmoA*) genes, which encode key enzymes of methanogens and methanotrophs, respectively (Yuan et al., 2009; Shrestha et al., 2010; Lee et al., 2014).

It is important to acknowledge that methane is a metabolic end product produced in deep soil horizons that diffuses upward toward the narrow oxic surface zone where aerobic methanotrophs flourish. Focusing on the deep soil horizons that are the source of methane (**Figures 7C,D**), the absolute abundances of methanogens were clearly higher in the rhizosphere than in the bulk soil above 200 mm depth; this is consistent with higher plant-derived TOC in the rhizosphere (**Figure 1B**) fueling fermentative food chains whose final step is mediated by methanogenic populations. Methanogenic populations clearly enriched in the rhizosphere soil at the depths of 200, 100, 60, and 30 mm depths (relative to the bulk soil) included *Methanosaeta*, *Methanoregula*, *Methanocella*, *Methanobacterium*, *Methanosphaerula*, and GOM_Arc_I; these genera are candidates for *in situ* methanogenesis that may have led to the high concentration of rhizosphere methane at the 9 mm depth (**Figure 1A**). The most obvious trend in the absolute abundances of the methanotrophic populations is the fact that total numbers were enriched at deeper depths (especially 6–7 and 7–8 mm) in the rhizosphere soil (**Figures 7A,B**). This enrichment is consistent with the notion that higher methanogenic populations in the deep rhizosphere (relative to bulk) soils lead to higher concentrations and fluxes of methane; thus, methanotropic populations likely were responding to that flux at deeper depths. Particular populations enhanced at the 6–8 mm depth (relative to the same depths in the bulk soil profile) included: *Methylosarcina, Methylococcus, Methylosinus,* and unclassified *Methylocystaceae*; these genera are candidates

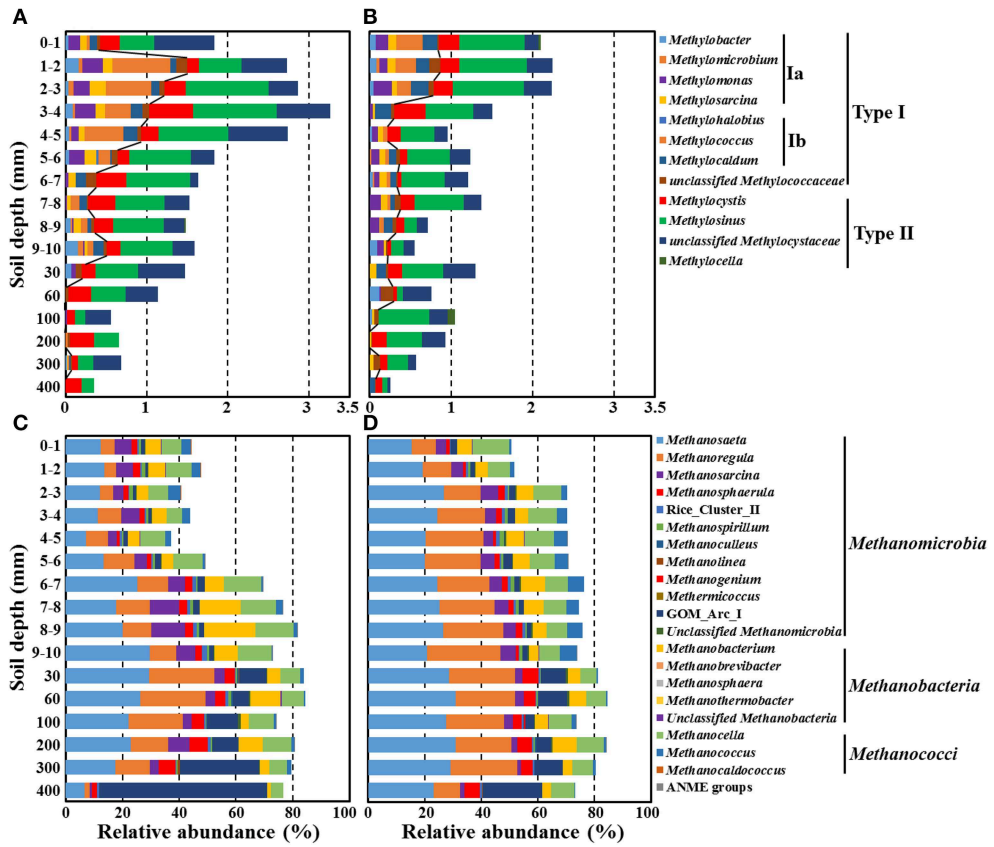

FIGURE 6 | Relative abundances of putative methanotrophs (A,B) and methanogens (C,D) classified at the genus level along a depth gradient in the bulk (A,C) and rhizosphere (B,D) soils of the rice paddy. The lines in **(A,B)** indicate the boundaries between type I (left) and type II (right) methanotrophs. GOM_Arc_I; Gulf of Mexico_Archaea_I.

for *in situ* methanotrophy. It must be acknowledged that another key factor likely influencing the abundances of methanotrophs in the rhizosphere vs. soils shown in **Figures 7A,B** may be channels formed in the soil by rice roots and their secondary impacts on oxygen and methane distribution (Kumaraswamy et al., 1997; Eller et al., 2005; Kerdchoechuen, 2005; Gutierrez et al., 2014).

Discussion

Methane production and oxidation are expected to vary with soil depth and methane is expected to be transported in flooded rice paddies because the soil-surface region alone is aerobic in the flooded rice paddies. However, to date, only few studies on the microbial communities along a depth gradient in rice paddies have been performed, and these did not investigate methanotrophs (Lüdemann et al., 2000; Noll et al., 2005). Recently, the bacterial methanotroph communities at the soil surface and the archaeal methanogen communities in anaerobic regions have been investigated by using T-RFLP and denaturing gradient gel electrophoresis, respectively (Watanabe et al., 2010; Reim et al., 2012). In these studies, no analysis of the concomitant methane concentrations along a depth gradient in the rice paddies was performed, which made it impossible to accurately trace the methane metabolic processes in the rice paddies. Therefore, in this study, we investigated the communities of methanotrophs as well as methanogens using parallel 454-pyroseqeuncing along a depth gradient comprising the surface and anaerobic regions, along with the analysis of oxygen, methane, and TOC concentrations and methanogenic and methanotrophic abundances, in the bulk and rhizosphere soils of the flooded rice paddy.

The methane and TOC concentrations and the methanogen abundances were clearly higher in the rhizosphere soil than in the bulk soil (**Figures 1**, **7**), suggesting that the organic carbon for methane production are derived mainly from the root exudates of the rice plants, as reported previously (Lu et al., 2000; Kimura et al., 2004; Lu and Conrad, 2005; Yuan et al., 2012; Pump et al., 2015). Bolstering the notion that *in situ* methane production and flux was high in the rhizosphere was the observation (**Figure 1A**) that at 9 mm, the methane concentration in the rhizosphere soil was nearly double that at the same depth in the bulk soil. The vertical methane concentration and methanogen abundance profiles indicated that methane is maximally produced at 30–200-mm depth in the rhizosphere soil (**Figures 1**, **7**), while the vertical oxygen concentration and methanotroph abundance profiles suggested that methane is metabolized primarily aerobically at

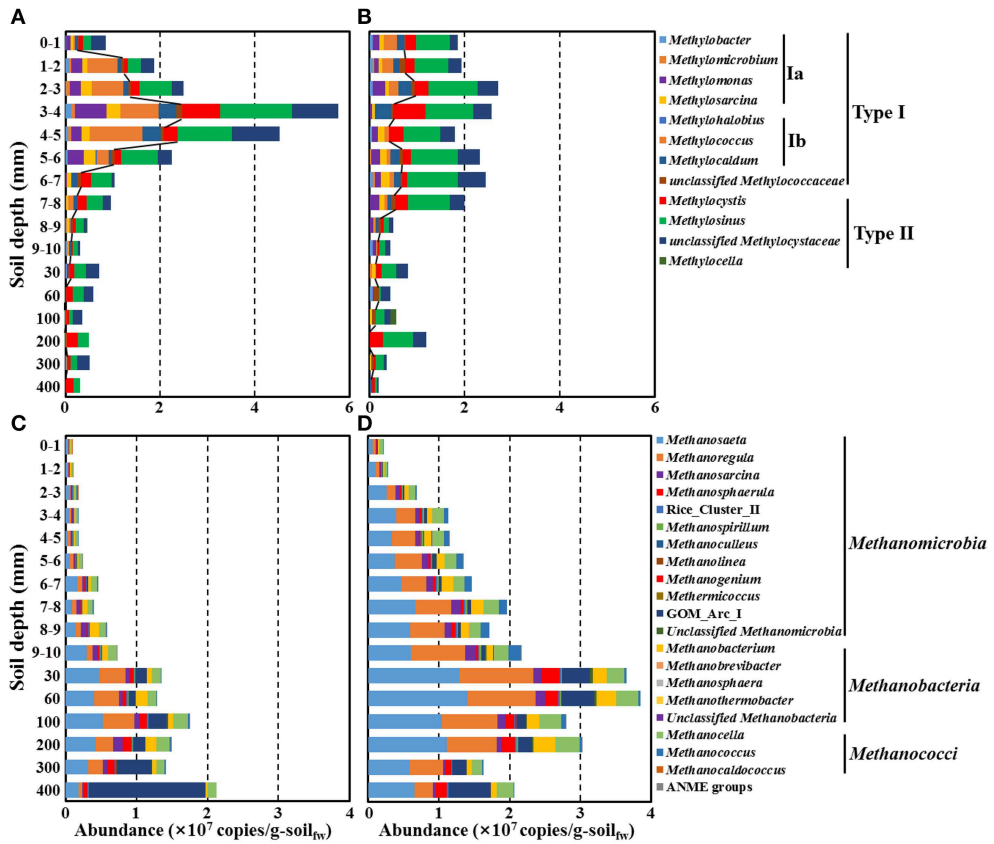

FIGURE 7 | Estimated absolute abundances of putative methanotrophs (A,B) and methanogens (C,D) along a depth gradient in the bulk (A,C) and rhizosphere (B,D) soils of the rice paddy. The absolute abundances (16S rRNA gene copy numbers) of methanotrophs and methanogens were estimated by multiplication of the relative abundances in **Figure 6** and the corresponding 16S rRNA gene copy numbers of *Bacteria* and *Archaea* in **Figure 2**. The lines in **(A,B)** indicate the boundaries between type I (inside) and type II (outside) methanotrophs. g-soil$_{fw}$, gram-soil fresh weight.

the oxic-anoxic interface in the rice paddy, where both methane and oxygen are supplied (**Figures 1**, **7**); ANME sequences were scarcely detected in the rice paddy soil. These results suggested that methane is produced mainly from organic carbon derived from the rice roots at 30–200-mm depth in the rhizosphere soil and diffuses into the bulk and surface soil layers, and is oxidized mainly at the oxic-anoxic interface. It has been reported that oxygen is released through the aerenchyma of plant roots and that a slightly aerobic condition can occur at the rhizosphere of rice plants (Lu et al., 2000; Shrestha et al., 2008; Blossfeld et al., 2011; Ma et al., 2013). However, in this study, no clear differences in the oxygen concentrations in the bulk and rhizosphere soils were observed (**Figure 1A**). The total bacterial abundances were distinctly higher in the rhizosphere soil than in the bulk soil (**Figure 2A**). Moreover, the relative abundance of methanotrophs in the rhizosphere soil was clearly lower than those in the bulk soil (**Figures 6A,B**), indicating that bacteria other than methanotrophs were enriched in the rhizosphere. These results might be explained by the exudation of organic matter from rice plant roots that increased the growth of heterotrophic bacteria, causing rapid consumption of oxygen near the rice roots.

It has been reported that methanotrophs from distinct taxonomic groups are influenced differently by oxygen and methane concentrations (Henckel et al., 2000; Lüke and Frenzel, 2011; Reim et al., 2012; Lüke et al., 2014), indicating that the methanotroph abundance and composition can differ along a depth gradient in rice paddies. The abundances of the methanotrophs were observed to peak around the oxic-anoxic interface where both oxygen and methane are present, which was consistent with the results of a previous study on methanotrophic activities based on *pmoA* transcript levels and oxygen respiration at the oxic-anoxic interface (Reim et al., 2012). The study by Reim et al. (2012) showed that an alternating pattern of predominance of type Ia and type II methanotrophs occurred at the surface and that type Ia methanotrophs were predominant around the oxic-anoxic interface. However, in the current study, no alternating pattern of type Ia and type II methanotrophs was observed around the oxic-anoxic interface, which might be explained by the less distinct stratification in oxygen and methane gradients in the planted rice paddy or by differences in environmental features. Instead, type Ib methanotrophs dominated the type Ia methanotrophs at the oxic-anoxic interface. The abundances of

type I methanotrophs such as *Methylococcus*, *Methylomonas*, and *Methylocaldum* decreased more obviously compared to type II methanotrophs with increasing depth of the rice paddy, while the abundances of type II methanotrophs such as *Methylocystis* and *Methylosinus* were relatively constant along a depth gradient (**Figures 6A,B**), which suggests that type I methanotrophs require high oxygen availability to oxidize methane, while type II methanotrophs can oxidize methane more efficiently under limited oxygen availability. These results were consistent with those of previous studies, which showed that type I methanotrophs generally display high methane-oxidizing activity under high oxygen and limited methane availability compared to type II methanotrophs (Henckel et al., 2000; MaCalady et al., 2002; Knief and Dunfield, 2005; Shrestha et al., 2008; Wu et al., 2009).

The fact that methanogens are obligate anaerobes and that the organic carbon for methane production is derived mainly from root exudates in rice paddies may explain why the methanogen abundances gradually increased with depth and peaked in the rhizosphere soil at 30–200 mm depth (**Figures 6**, **7**). *Methanosaeta* and *Methanoregula*, known as acetoclastic and hydrogenotrophic methanogens, respectively, were predominant in the rice paddy, which suggests that hydrogen as well as acetate are important substrates for methanogenesis in rice paddies. In particular, the abundances of *Methanoregula* increased rapidly along a depth gradient, suggesting that the hydrogen concentration increases with depth (Bräuer et al., 2006). Although the methane concentrations were very low below 300-mm depth (**Figure 1A**), the absolute abundances of populations classified as the methanogens were high due to the presence of GOM_Arc_I-related archaea (**Figures 7C,D**). Although the GOM_Arc_I sequences were classified within *Methanomicrobia* in this study, the GOM_Arc_I, which was first identified from methane hydrate-rich sediments in the Gulf of Mexico, was originally presumed as ANME-2d because their 16S rRNA gene sequences were also phylogenetically related to those of ANME-2d (Mills et al., 2003, 2005). However, to date, no study establishing physiological function of GOM_Arc_I as methanogens or ANME has been published. In the current study, the abundances of GOM_Arc_I rapidly increased with decreasing methane concentrations, which supports the notion, in rice paddy soils, the GOM_Arc_I may be heterotrophic archaea; corroborated by the high TOC concentrations below 300-mm depth (**Figures 1**, **7**).

In this investigation, we combined the millimeter-scale sampling of oxygen, methane, TOC concentrations, together with pyrosequencing-based community characterization of communities, especially methanotrophs and methanogens, in both bulk and rhizosphere soils of a planted rice paddy. By focusing on absolute abundances of both methanogenic and methanotrophic genera (**Figures 7C,D** and **Figures 7A,B**, respectively), we found contrasts between methanogens and methanotrophs at depths sampled across rhizosphere and bulk soils that helped explain drastic methane depletion at the 4–10 mm depths in both bulk and rhizosphere soils and a methane concentration at 9 mm in the rhizosphere soil nearly double that that of the bulk soil. As a result we hypothesize that populations of methanogens (*Methanosaeta*, *Methanoregula*, *Methanocella*, *Methanobacterium*, and *Methanosphaerula*) and methanotrophs (*Methylosarcina, Methylococcus, Methylosinus,* and unclassified *Methylocystaceae*) likely were physiologically active *in situ*.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00639

References

Biddle, J. F., Cardman, Z., Mendlovitz, H., Albert, D. B., Lloyd, K. G., Boetius, A., et al. (2012). Anaerobic oxidation of methane at different temperature regimes in Guaymas Basin hydrothermal sediments. *ISME J.* 6, 1018–1031. doi: 10.1038/ismej.2011.164

Blossfeld, S., Gansert, D., Thiele, B., Kuhn, A. J., and Lösch, R. (2011). The dynamics of oxygen concentration, pH value, and organic acids in the rhizosphere of *Juncus* spp. *Soil Biol. Biochem.* 43, 1186–1197. doi: 10.1016/j.soilbio.2011.02.007

Bräuer, S., Cadillo-Quiroz, H., Yashiro, E., Yavitt, J. B., and Zinder, S. H. (2006). Isolation of a novel acidiphilic methanogen from an acidic peat bog. *Nature* 442, 192–194. doi: 10.1038/nature04810

Breidenbach, B., and Conrad, R. (2015). Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Front. Microbiol.* 5:752. doi: 10.3389/fmicb.2014.00752

Bridgham, S. D., Cadillo-Quiroz, H., Keller, J. K., and Zhuang, Q. (2013). Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. *Glob. Change Biol.* 19, 1325–1346. doi: 10.1111/gcb.12131

Chao, A. (1987). Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* 43, 783–791. doi: 10.2307/2531532

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

Conrad, R. (2002). Control of microbial methane production in wetland rice fields. *Nutr. Cycl. Agroecosyst.* 64, 59–69. doi: 10.1023/A:1021178713988

Conrad, R. (2007). Microbial ecology of methanogens and methanotrophs. *Adv. Agron.* 96, 1–63. doi: 10.1016/s0065-2113(07)96005-8

Costa, K. C., and Leigh, J. A. (2014). Metabolic versatility in methanogens. *Curr. Opin. Biotechnol.* 29, 70–75. doi: 10.1016/j.copbio.2014.02.012

Denman, S. E., and McSweeney, C. S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58, 572–582. doi: 10.1111/j.1574-6941.2006.00190.x

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 21, 494–504. doi: 10.1093/bioinformatics/btr381

Eller, G., Krüger, M., and Frenzel, P. (2005). Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in

paddy soil. *FEMS Microbiol. Ecol.* 51, 279–291. doi: 10.1016/j.femsec.2004.09.007

Ettwig, K. F., Butler, M. K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M. M., et al. (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 464, 543–548. doi: 10.1038/nature08883

Felsenstein, J. (2002). *PHYLIP (Phylogeny Inference Package), Version 3.6a*. Seattle, WA: Department of Genetics, University of Washington.

Gilbert, B., and Frenzel, P. (1998). Rice roots and CH_4 oxidation: the activity of bacteria, their distribution and the microenvironment. *Soil Biol. Biochem.* 14, 1903–1916. doi: 10.1016/S0038-0717(98)00061-3

Groot, T. T., van Bodegom, P. M., Harren, F. J. M., and Meijer, H. A. J. (2003). Quantification of methane oxidation in the rice rhizosphere using ^{13}C-labelled methane. *Biogeochemistry* 64, 355–372. doi: 10.1023/A:1024921714852

Gutierrez, J., Atulba, S. L., Kim, G., and Kim, P. J. (2014). Importance of rice root oxidation potential as a regulator of CH_4 production under waterlogged conditions. *Biol. Fertil. Soils* 50, 861–868. doi: 10.1007/s00374-014-0904-0

Haroon, M. F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., et al. (2013). Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 500, 567–570. doi: 10.1038/nature12375

Henckel, T., Roslev, P., and Conrad, R. (2000). Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. *Environ. Microbiol.* 2, 666–679. doi: 10.1046/j.1462-2920.2000.00149.x

Herman, D., and Roberts, D. (2006). The influence of structural components of alkyl esters on their anaerobic biodegradation in marine sediment. *Biodegradation* 17, 457–463. doi: 10.1007/s10532-005-9016-8

IPCC. (2007). "Climate change 2007: the physical science basis," in *Working Group I Contribution to the IPCC Fourth Assessment Report*. Cambridge; New York: Cambridge University Press.

Jung, J. Y., Lee, S. H., Kim, J. M., Park, M. S., Bae, J. W., Hahn, Y., et al. (2011). Metagenomic analysis of kimchi, a traditional Korean fermented food. *Appl. Environ. Microbiol.* 77, 2264–2274. doi: 10.1128/AEM.02157-10

Kerdchoechuen, O. (2005). Methane emission in four rice varieties as related to sugars and organic acids of roots and root exudates and biomass yield. *Agric. Ecosyst. Environ.* 108, 155–163. doi: 10.1016/j.agee.2005.01.004

Kimura, M., Murase, J., and Lu, Y. (2004). Carbon cycling in rice field ecosystems in the context of input, decomposition and translocation of organic materials and the fates of their end products (CO_2 and CH_4). *Soil Biol. Biochem.* 36, 1399–1416. doi: 10.1016/j.soilbio.2004.03.006

Knief, C., and Dunfield, P. F. (2005). Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. *Environ. Microbiol.* 7, 1307–1317. doi: 10.1111/j.1462-2920.2005.00814.x

Krause, S., Lüke, C., and Frenzel, P. (2010). Succession of methanotrophs in oxygen-methane counter-gradients of flooded rice paddies. *ISME J.* 4, 1603–1607. doi: 10.1038/ismej.2010.82

Kumaraswamy, S., Ramakrishnan, B., Satpathy, S. N., Rath, A. K., Misra, S., Rao, V. R., et al. (1997). Spatial distribution of methane-oxidizing activity in a flooded rice soil. *Plant Soil* 191, 241–248. doi: 10.1023/A:1004274302326

Lee, H. J., Jung, J. Y., Oh, Y. K., Lee, S. S., Madsen, E. L., and Jeon, C. O. (2012). Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and ^{1}H nuclear magnetic resonance spectroscopy. *Appl. Environ. Microbiol.* 78, 5983–5993. doi: 10.1128/AEM.00104-12

Lee, H. J., Kim, S. Y., Kim, P. S., Madsen, E. L., and Jeon, C. O. (2014). Methane emission and dynamics of methanotrophic and methanogenic communities in a flooded rice field ecosystem. *FEMS Microbiol. Ecol.* 88, 195–212. doi: 10.1111/1574-6941.12282

Liesack, W., Schnell, S., and Revsbech, N. P. (2000). Microbiology of flooded rice paddies. *FEMS Microbiol. Rev.* 24, 624–645. doi: 10.1111/j.1574-6976.2000.tb00563.x

Liu, D., Ishikawa, H., Nishida, M., Tsuchiya, K., Takahashi, T., Kimura, M., et al. (2015). Effect of paddy-upland rotation on methanogenic archaeal community structure in paddy field soil. *Microb. Ecol.* 69, 160–168. doi: 10.1007/s00248-014-0477-3

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lüdemann, H., Arth, I., and Liesack, W. (2000). Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* 66, 754–762. doi: 10.1128/AEM.66.2.754-762.2000

Lüke, C., and Frenzel, P. (2011). Potential of *pmoA* amplicon pyrosequencing for methanotroph diversity studies. *Appl. Environ. Microbiol.* 77, 6305–6309. doi: 10.1128/AEM.05355-11

Lüke, C., Frenzel, P., Ho, A., Fiantis, D., Schad, P., Schneider, B., et al. (2014). Macroecology of methane-oxidizing bacteria: the β-diversity of *pmoA* genotypes in tropical and subtropical rice paddies. *Environ. Microbiol.* 16, 72–83. doi: 10.1111/1462-2920.12190

Lu, Y., and Conrad, R. (2005). *In situ* stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309, 1088–1090. doi: 10.1126/science.1113435

Lu, Y., Wassmann, R., Neue, H. U., and Huang, C. Y. (2000). Dynamics of dissolved organic carbon and methane emissions in a flooded rice soil. *Soil Sci. Soc. Am. J.* 6, 2011–2017. doi: 10.2136/sssaj2000.6462011x

Ma, K., Conrad, R., and Lu, Y. (2012). Responses of methanogen *mcrA* genes and their transcripts to an alternate dry/wet cycle of paddy field soil. *Appl. Environ. Microbiol.* 78, 445–454. doi: 10.1128/AEM.06934-11

Ma, K., Conrad, R., and Lu, Y. (2013). Dry/wet cycles change the activity and population dynamics of methanotrophs in rice field soil. *Appl. Environ. Microbiol.* 79, 4932–4939. doi: 10.1128/AEM.00850-13

Ma, K., and Lu, Y. (2011). Regulation of microbial methane production and oxidation by intermittent drainage in rice field soil. *FEMS Microbiol. Ecol.* 75, 446–456. doi: 10.1111/j.1574-6941.2010.01018.x

Ma, K., Qiu, Q., and Lu, Y. (2010). Microbial mechanism for rice variety control on methane emission from rice field soil. *Glob. Change Biol.* 16, 3085–3095. doi: 10.1111/j.1365-2486.2009.02145.x

MaCalady, J. L., McMillan, A. M. S., Dickens, A. F., Tyler, S. C., and Scow, K. M. (2002). Population dynamics of type I and II methanotrophic bacteria in rice soil. *Environ. Microbiol.* 4, 148–157. doi: 10.1046/j.1462-2920.2002.00278.x

Mills, H. J., Hodges, C., Wilson, K., MacDonald, I. R., and Sobecky, P. A. (2003). Microbial diversity in sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico. *FEMS Microbiol. Ecol.* 46, 39–52. doi: 10.1016/S0168-6496(03)00191-0

Mills, H. J., Martinez, R. J., Story, S., and Sobecky, P. A. (2005). Characterization of microbial community structure in Gulf of Mexico gas hydrates: comparative analysis of DNA- and RNA-derived clone libraries. *Appl. Environ. Microbiol.* 71, 3235–3247. doi: 10.1128/AEM.71.6.3235-3247.2005

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382–395. doi: 10.1111/j.1462-2920.2005.00700.x

Op den Camp, H. J., Islam, T., Stott, M. B., Harhangi, H. R., Hynes, A., Schouten, S., et al. (2009). Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ. Microbiol. Rep.* 1, 293–306. doi: 10.1111/j.1758-2229.2009.00022.x

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Pump, J., Pratscher, J., and Conrad, R. (2015). Colonization of rice roots with methanogenic archaea controls photosynthesis-derived CH_4 emission. *Environ. Microbiol.* doi: 10.1111/1462-2920.12675. [Epub ahead of print].

Reim, A., Lüke, C., Krause, S., Pratscher, J., and Frenzel, P. (2012). One millimeter makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic-anoxic interface in a flooded paddy soil. *ISME J.* 6, 2128–2139. doi: 10.1038/ismej.2012.57

Revsbech, N. P., Pedersen, O., Reichardt, W., and Briones, A. (1999). Microsensor analysis of oxygen and pH in the rice rhizosphere under field and laboratory conditions. *Biol. Fertil. Soils* 29, 379–385. doi: 10.1007/s003740050568

Roesch, L. F. W., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K. M., Kent, A. D., et al. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J.* 1, 283–290. doi: 10.1038/ismej.2007.53

Sakai, S., Imachi, H., Sekiguchi, Y., Tseng, I. C., Ohashi, A., Harada, H., et al. (2009). Cultivation of methanogens under low-hydrogen conditions by using the coculture method. *Appl. Environ. Microbiol.* 75, 4892–4896. doi: 10.1128/AEM.02835-08

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Semrau, J. D., DiSpirito, A. A., and Yoon, S. (2010). Methanotrophs and copper. *FEMS Microbiol. Rev.* 34, 496–531. doi: 10.1111/j.1574-6976.2010.00212.x

Shannon, C. E., and Weaver, W. (1963). *The Mathematical Theory of Communication*. Urbana, IL: University of Illinois Press.

Shen, L. D., Liu, S., Huang, Q., Lian, X., He, Z. F., Geng, S., et al. (2014). Evidence for the cooccurrence of nitrite-dependent anaerobic ammonium and methane oxidation processes in a flooded paddy field. *Appl. Environ. Microbiol.* 80, 7611–7619. doi: 10.1128/AEM.02379-14

Shimane, Y., Hatada, Y., Minegishi, H., Echigo, A., Nagaoka, S., Miyazaki, M., et al. (2011). Salarchaeum japonicum gen. nov., sp. nov., an aerobic, extremely halophilic member of the Archaea isolated from commercial salt. *Int. J. Syst. Evol. Microbiol.* 61, 2266–2270. doi: 10.1099/ijs.0.025064-0

Shrestha, M., Abraham, W. R., Shrestha, P. M., Noll, M., and Conrad, R. (2008). Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids. *Environ. Microbiol.* 10, 400–412. doi: 10.1111/j.1462-2920.2007.01462.x

Shrestha, M., Shrestha, P. M., Frenzel, P., and Conrad, R. (2010). Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. *ISME J.* 4, 1545–1556. doi: 10.1038/ismej.2010.89

Takai, K., and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl. Environ. Microbiol.* 66, 5066–5072. doi: 10.1128/AEM.66.11.5066-5072.2000

Wainø, M., Tindall, B. J., and Ingvorsen, K. (2000). Halorhabdus utahensis gen. nov., sp. nov., an aerobic, extremely halophilic member of the Archaea from Great Salt Lake, Utah. *Int. J. Syst. Evol. Microbiol.* 50, 183–190. doi: 10.1099/00207713-50-1-183

Watanabe, T., Wang, G., Taki, K., Ohashi, Y., Kimura, M., and Asakawa, S. (2010). Vertical changes in bacterial and archaeal communities with soil depth in Japanese paddy fields. *Soil Sci. Plant Nutr.* 56, 705–715. doi: 10.1111/j.1747-0765.2010.00511.x

Wu, L., Ma, K., and Lu, Y. (2009). Rice roots select for type I methanotrophs in rice field soil. *Syst. Appl. Microbiol.* 32, 421–428. doi: 10.1016/j.syapm.2009.05.001

Yuan, Q., Pump, J., and Conrad, R. (2012). Partitioning of CH_4 and CO_2 production originating from rice straw, soil and root organic carbon in rice microcosms. *PLoS ONE* 7:e49073. doi: 10.1371/journal.pone.0049073

Yuan, Y., Conrad, R., and Lu, Y. (2009). Responses of methanogenic archaeal community to oxygen exposure in rice field soil. *Environ. Microbiol. Rep.* 1, 347–354. doi: 10.1111/j.1758-2229.2009.00036.x

Yuan, Y., Conrad, R., and Lu, Y. (2011). Transcriptional response of methanogen *mcrA* genes to oxygen exposure of rice field soil. *Environ. Microbiol. Rep.* 3, 320–328. doi: 10.1111/j.1758-2229.2010.00228.x

Zhou, J., Wu, L., Deng, Y., Zhi, X., Jian, Y. H., Tu, Q., et al. (2011). Reproducibility and quantitation of amplicon sequencing-based detection. *ISME J.* 5, 1303–1313. doi: 10.1038/ismej.2011.11

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REVIEW
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Ecology of Nitrogen Fixing, Nitrifying, and Denitrifying Microorganisms in Tropical Forest Soils

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Soil microorganisms play important roles in nitrogen cycling within forest ecosystems. Current research has revealed that a wider variety of microorganisms, with unexpected diversity in their functions and phylogenies, are involved in the nitrogen cycle than previously thought, including nitrogen-fixing bacteria, ammonia-oxidizing bacteria and archaea, heterotrophic nitrifying microorganisms, and anammox bacteria, as well as denitrifying bacteria, archaea, and fungi. However, the vast majority of this research has been focused in temperate regions, and relatively little is known regarding the ecology of nitrogen-cycling microorganisms within tropical and subtropical ecosystems. Tropical forests are characterized by relatively high precipitation, low annual temperature fluctuation, high heterogeneity in plant diversity, large amounts of plant litter, and unique soil chemistry. For these reasons, regulation of the nitrogen cycle in tropical forests may be very different from that of temperate ecosystems. This is of great importance because of growing concerns regarding the effect of land use change and chronic-elevated nitrogen deposition on nitrogen-cycling processes in tropical forests. In the context of global change, it is crucial to understand how environmental factors and land use changes in tropical ecosystems influence the composition, abundance and activity of key players in the nitrogen cycle. In this review, we synthesize the limited currently available information regarding the microbial communities involved in nitrogen fixation, nitrification and denitrification, to provide deeper insight into the mechanisms regulating nitrogen cycling in tropical forest ecosystems. We also highlight the large gaps in our understanding of microbially mediated nitrogen processes in tropical forest soils and identify important areas for future research.

Keywords: nitrogen processes, tropical forest soils, soil microbial community, nitrogen functional genes, nitrous oxide

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INTRODUCTION

Nitrogen (N) is an important component of proteins and nucleic acids, and thus is an essential nutrient for all organisms. Furthermore, the N supply to organisms regulates primary productivity in many natural ecosystems. N exists in multiple oxidation states and chemical forms in nature, and transformations among these different forms are typically catalyzed by microorganisms (Hayatsu et al., 2008). Soil microorganisms constitute a major portion of the biodiversity in soils and play a pivotal role in soil processes (Fierer and Jackson, 2006; Schimel and Schaeffer, 2012;

Philippot et al., 2013), which ultimately affect the functioning of terrestrial ecosystems. Given the biogeochemical contributions of microbes, microbial community dynamics may be associated with temporal and spatial variation in N processes and rates. Thus, understanding the link between biogeochemical N processes and microbial community dynamics can provide a more mechanistic understanding of the N cycle than the direct observation of N dynamics. Likewise, it is important to know the factors that influence the biodiversity and functioning of soil microbes that mediate these processes in order to predict ecosystem responses to a changing environment (Singh et al., 2010).

Tropical forests are particularly important in the N cycle. Although these ecosystems occupy only 12% of the Earth's surface, they contribute ∼70% of terrestrial N fixation and emit ∼50% of nitrous oxide (N_2O; Townsend et al., 2011). They are a major reservoir of biodiversity and play a vital role in regulating the Earth's climate and biogeochemical cycles through their vast exchanges of energy, water, and nutrients with the atmosphere (Foley et al., 2003; Bonan, 2008). Tropical forests account for nearly 40% of terrestrial net primary production, contain 25% of the world's biomass carbon (C) and play a critical role in buffering the atmosphere against rising carbon dioxide (CO_2) (Townsend et al., 2011; Wright, 2013). On the other hand, these forest ecosystems are threatened by high levels of deforestation, rapid rates of land conversion to agriculture, and chronic-elevated N deposition, with important implications for global climate (Stork et al., 2009; Hietz et al., 2011). As a result, there have been increased efforts recently to understand and predict how soil microbial communities in tropical forests regulate biogeochemical processes, in particular N cycling.

Our understanding of biological N cycling in forest ecosystems has undergone a major shift in the past decade in concert with the growing interest in integrating evolutionary and ecological theory into microbial ecology (Prosser et al., 2007; Fierer et al., 2009b; Philippot et al., 2010), the rapid development of molecular-based culture-independent methods (Roesch et al., 2007; Caporaso et al., 2011; Palmer et al., 2012), and the use of N isotope tracer techniques (Baggs, 2008; Kool et al., 2010). These advances have increased our understanding of microbial communities, their functional potential, and their physiological state (Houlton et al., 2006; Roesch et al., 2007; Levy-Booth et al., 2014), and have sparked a new interest in the functional link between soil microbial community structure and N cycling (Cusack et al., 2011; Treseder et al., 2011; Philippot et al., 2013). As a result, we are beginning to understand the microbial community ecology involved in N processes in forest ecosystems, which provide us with a more complete picture of the regulation of the N cycle and its response to environmental change (Bottomley et al., 2012; Levy-Booth et al., 2014; Zhang et al., 2014).

Although some excellent reviews of the ecology of N-cycling microorganisms in soils exist (e.g., Wallenstein et al., 2006; Philippot et al., 2007; Hayatsu et al., 2008; Braker and Conrad, 2011; Levy-Booth et al., 2014), these focus primarily on temperate systems, and the relationship between N processes and microbial community dynamics in tropical forest soils remains poorly understudied. In this review we describe and summarize currently available information regarding the relationship between soil microbial ecology and three relevant N-cycling processes that govern the fate of N in tropical forest soils. Although we also briefly introduce other important microbial N processes, we focus on the N-fixer (diazotrophs), nitrifier and denitrifier communities because these functional groups have been the most studied in tropical soils. We close our review with a discussion of the impact of anthropogenic environmental changes on the microbially mediated mechanisms regulating N cycling in tropical forest ecosystems.

THE NITROGEN CYCLE IN TROPICAL FOREST SOILS

Tropical soils are generally on older geologic substrates depleted of rock-derived elements such as phosphorus (P) and cations. As a consequence, these soils are typically acidic (pH < 5) and their fertility is often dependent on the cycling of a thin layer of organic matter associated with the large amount of plant litter material (Hall and Matson, 2003; Macrae et al., 2013). Thus, in contrast to temperate forests, tropical forests on highly weathered soils are assumed to be primarily limited by P and "N-saturated" (Hedin et al., 2009; Cleveland et al., 2011), leading to an open dynamic N cycle, with relatively high levels of N loss via denitrification and leaching (Vitousek and Matson, 1988; Martinelli et al., 1999; Brookshire et al., 2012) balanced by high levels of N fixation (Reed et al., 2007; Cusack et al., 2009). This assumption is also supported by observations of high plant and soil N/P ratios, high abundance and diversity of legume plants, and the export of bioavailable N at the ecosystem scale (Supplemenatary Table S1; Houlton et al., 2006; Hedin et al., 2009; Vitousek et al., 2010). For example, some fertilization experiments conducted in tropical forests have demonstrated a stronger response of plant biomass to added P than to added N, supporting the paradigm that tropical ecosystems on weathered soils are predominantly P limited (Tanner et al., 1998; Harrington et al., 2001). However, there is emerging evidence of limitation and colimitation by other nutrients, such as N and potassium, in tropical forests (Kaspari et al., 2008; Wullaert et al., 2010; Wright et al., 2011). For instance, strong colimitation by N and P of vegetation and soil organisms has been observed in an Ecuadorian montane forest (Wullaert et al., 2010; Homeier et al., 2012). In this forest ecosystem, moderate additions of N (50 kg ha^{-1} yr^{-1}) and P (10 kg ha^{-1} yr^{-1}) led to not only an increase in foliar N and P concentrations, but also altered soil microbial biomass, standing fine root biomass, stem growth, and litterfall (Homeier et al., 2012). Additionally, forest disturbance may lead to N limitation of young secondary forests (Davidson et al., 2007). Research to date suggests that nutrient limitation in these ecosystems is likely not a question of N vs. P, but instead is the result of complex interactions among multiple nutrient cycles and their linkages with biological processes (Townsend et al., 2011; Alvarez-Clare et al., 2013).

Soil N transformations in tropical forests are affected by unique soil characteristics (e.g., low pH, rapidly fluctuating

redox conditions and large amounts of Fe oxides, plant litter material and available N content) and environmental conditions (e.g., high humidity and low annual fluctuation in temperature), making it difficult to identify the main pathways and mechanisms affecting process-specific transformations (Supplementary Table S1; Vitousek and Matson, 1988; Hedin et al., 2009; Xu et al., 2013; Gao et al., 2015). For example, resource stoichiometry influences microbial growth rates (Rousk and Bååth, 2007) and the production of extracellular enzymes that degrade soil organic matter (Mooshammer et al., 2012). However, it remains unclear if the pattern of nutrient limitation documented for plants (i.e., strong limitation by P) in tropical forests holds true for soil microbes. For instance, there is evidence that P can limit microbial growth (Turner and Joseph Wright, 2014) and mineralization (Cleveland and Townsend, 2006; Kaspari et al., 2008) in lowland tropical forests found on heavily weathered soils. In contrast, soil microbial biomass and N mineralization in tropical montane forests on younger soils seem to be constrained primarily by N limitation (Hall and Matson, 2003; Cusack et al., 2010). Furthermore, it has been shown that the response of soil microorganisms to nutrient addition in an Ecuadorian montane rainforest varies with both altitude and duration of nutrient addition (Krashevska et al., 2014). Thus, nutrient availability to soil tropical communities can be affected by multiple factors such as elevation, temperature, rainfall and differences in parent material.

The complexity of the biological N cycle in tropical forest soils could be depicted by a simplified model (**Figure 1**) that consists of three main processes (Levy-Booth et al., 2014): (1) Decomposition of plant litter and dead organisms to soil organic matter, which can be further degraded to dissolved organic N and ammonium (NH_4^+); (2) assimilative processes of dissolved organic N, nitrate (NO_3^-) and NH_4^+ by plants and microorganisms for growth and replication; and (3) dissimilative processes including N fixation, nitrification, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA), as well as newly described pathways such as codenitrification and anammox. The pool sizes and rates of production of N available to soil microbes and plants are much greater in many tropical forest soils than in temperate counterparts, due to high rates of N fixation by free-living microbes and rhizobia associated with legumes, which are especially abundant in many tropical ecosystems (Hedin et al., 2009). This high N availability can exceed the ecosystem N retention capacity and induce high rates of dissimilative processes, with N losses by NO_3^- leaching and emissions of nitric oxide (NO) and N_2O (Hall and Matson, 1999; Hedin et al., 2003; Biggs et al., 2004; Brookshire et al., 2012).

The N_2O released from soil is of particular concern because it is a potent greenhouse gas. Tropical forest soils are considered the largest natural source of N_2O with a source strength of 3.0 Tg N yr^{-1} (Werner et al., 2007a). The production of N_2O in soil has traditionally been assumed to result from the processes of nitrification and denitrification, but growing evidence suggests that other processes may be involved, including nitrifier denitrification (Wrage et al., 2001), non-denitrifying N_2O reduction (Sanford et al., 2012), DNRA (Tiedje, 1988), anaerobic ammonium oxidation (anammox; Mulder et al., 1995), and co-denitrification (Spott et al., 2011). Understanding the factors that determine the relative contributions of these different processes (and the microbes that mediate them) to N_2O flux has important implications for climate change. However, it is still not clear what underlying processes drive gaseous N production from tropical forest soils. For instance, it has been suggested that nitrification is an important source of N_2O in Amazon forest soils (Neill et al., 2005). However, some studies conducted in tropical forest ecosystems in Australia (Butterbach-Bahl et al., 2004), Kenya (Werner et al., 2007b), and Rwanda (Gharahi Ghehi et al., 2012) suggest that denitrification and nitrifier denitrification could be the main pathways of N_2O gas production rather than nitrification. Likewise, anammox bacteria have been detected in a wide range of soil environments (Humbert et al., 2010; Sonthiphand et al., 2014), but the role of these organisms in N loss from tropical forest soils has yet to be determined. The DNRA process has also been suggested as a possibly important pathway for NH_4^+ production in these soils (Silver et al., 2001), because the occurrence of DNRA is generally favored by low and fluctuating redox conditions, such as those in humid tropical forest soils (Pett-Ridge et al., 2006; Templer et al., 2008). For instance, Templer et al. (2008) found that the rate of DNRA (∼35% of the gross nitrification rate) in a humid tropical forest soil in Puerto Rico was much higher than the rate of N_2O production from denitrification.

N-CYCLING MICROBES IN TROPICAL FOREST SOILS

There is evidence that tropical forest soils may harbor novel microbial communities, such as unique clades of Acidobacteria (Kim et al., 2007) and Verrucomicrobia (Ranjan et al., 2015). However, these soils are an especially understudied microbial environment, and very few studies have explored the immense diversity of soil microorganisms in tropical forests (Kim et al., 2007; Bruce et al., 2010; Ranjan et al., 2015) or asked if they are phylogenetically or physiologically distinct from those in temperate soils (Fierer and Jackson, 2006; Fierer et al., 2012b; Ramirez et al., 2012). The majority of these studies have concentrated on the effects of land-use conversion on the structure and function of soil microbial communities (e.g., da Jesus et al., 2009; Lee-Cruz et al., 2013; Rodrigues et al., 2013; Paula et al., 2014; Ranjan et al., 2015). Few studies have examined seasonal influences of precipitation (Krashevska et al., 2012; McGuire et al., 2012), or the effects of plant species diversity (Carney and Matson, 2006; McGuire et al., 2012) or N addition (Cusack et al., 2010, 2011; Isobe et al., 2012; Liu et al., 2013a) on tropical soil microbial communities.

Linking microbial community composition, abundance, and activity to rates and controls of biogeochemical transformations is challenging in any ecosystem, but is especially challenging in tropical forest soils. This is due not only to the complexity of tropical forest microbial communities, but also to the high degree of heterogeneity in plant functional diversity and soil chemistry often found in tropical forest ecosystems. For example,

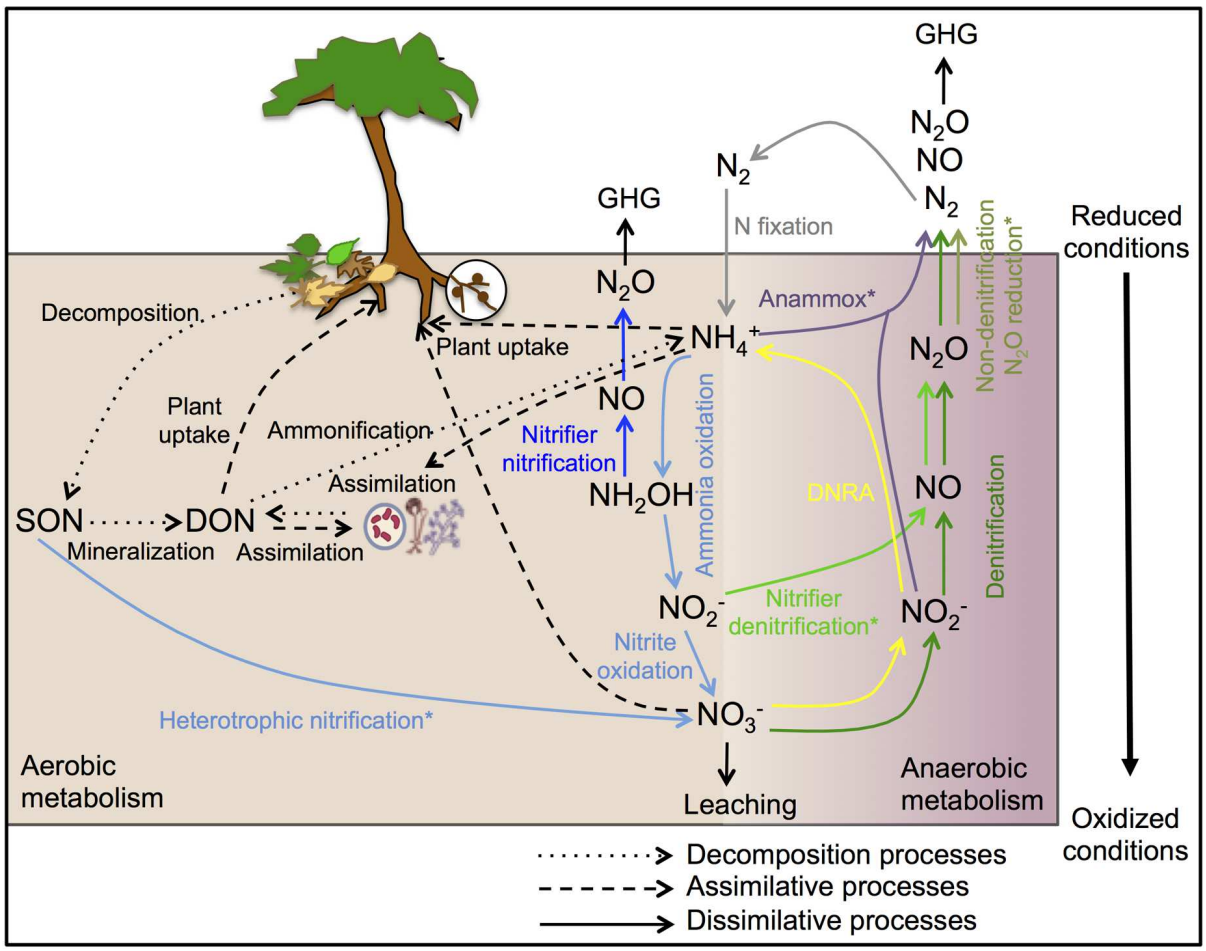

FIGURE 1 | Schematic representation of the biological N cycle in tropical forest soils. The arrows of decomposition, assimilative and abiotic processes are shown in black and the arrows of dissimilative processes are shown in different colors: N-fixation (gray), nitrification (light blue), nitrifier nitrification (dark blue), nitrifier denitrification (light green), denitrification (dark green), non-denitrification N_2O reduction (olive green), DNRA (yellow), and anammox (purple). DNRA, dissimilatory nitrate reduction to ammonium; SON, soil organic N; DON, dissolved organic N; GHG, greenhouse gas. *Occurrence and ecology of these processes in tropical forest soils has not been yet investigated.

rates of many microbially mediated N processes show marked substantial variation across small temporal and spatial scales in tropical forest soils (Silver et al., 2001; Davidson et al., 2004; Pett-Ridge et al., 2006). Tropical plant community composition varies strongly across space in many tropical forest ecosystems, and this can contribute to the high degree of heterogeneity in N cycle processes observed in these systems. For instance, the large amount of plant litter and rhizodeposition (the release of C compounds from living plant roots) in tropical forest soils may enhance microbial activity and stimulate production of microbial exoenzymes that "mine" for more complex soil organic N compounds (Paterson, 2003). The N cycle is also particularly susceptible to changes in soil redox conditions, which are highly variable in tropical forest soils and can cycle between oxic and anoxic on a scale of hours to days (Silver et al., 2001). These fluctuating soil redox regimes are a critical determinant of microbial metabolism (DeAngelis et al., 2010), and may facilitate the co-occurrence of microbial N transformations with significantly different sensitivities to soil O_2 availability, such as nitrification (oxic), denitrification (hypoxic), and DNRA (anoxic). Pett-Ridge et al. (2006) found that the rapidly fluctuating redox conditions in a humid tropical forest soil in Puerto Rico allow anoxic and oxic N processes to co-occur, which are mediated by tolerant and resilient microbial communities adapted to nutrient resources that are spatially and temporally variable.

A comprehensive understanding of the microbially mediated tropical N cycle is critical because it affects our knowledge of how tropical forests function within the integrated Earth-climate system. For example, quantification and characterization of microbial functional genes involved in N cycling (**Table 1** and Supplementary Table S2) can help create informative models of N-cycling rates and their feedback with C dynamics, as well as the impact of N_2O emissions from these soils, providing predictions of, and mitigation strategies for, greenhouse gas emissions (Levy-Booth et al., 2014; Lammel et al., 2015). We discuss below

TABLE 1 | Studies using N functional genes related to N fixation, nitrification, and denitrification processes in tropical forest soils.

N genes	Methods	Ecosystem	Edaphic characteristics	Condition tested	Major relationships in N functional genes	Reference
nifH, amoA, nirK, nirS, norB, nosZ	*In situ* measure of GHG, qPCR	PTF, pasture and crops in Amazonia (Brazil)	Clay Oxisol, pH 3.9–5.1, 0.23–0.29% TC, 0.01–0.02% TN	Influence of land use change on microbial gene abundance and GHG emissions	*nifH* and AOB are more abundant in forest soils. AOA dominates in all sites. *amoA* genes correlate with NO_3^-. PTF soils have the highest abundances of denitrifiers, which correlate with increased N_2O emissions	Lammel et al., 2015
nifH, amoA, nirS, nosZ	DGGE, qPCR	Humid TFs (Puerto Rico, USA)	Oxisol and Inceptisol, pH 4.1–6.3, 0.01–0.2% TC, 0.001–0.06% TN	Influence of parent material, forest types and soil depth on bacterial community structure and N functional genes	N genes decline significantly with soil depth. *nifH, nirS*, and *nosZ* are more abundant in the Inceptisol soil. *nirS* and *nosZ* have the highest abundance and AOB has the lowest abundance	Stone et al., 2015
amoA	^{15}N isotope technique (N transformations), qPCR	Evergreen subtropical forests (China)	Sandy loam Ferralsols and Cambisols, pH 4.2–5.8, 1.43–2.09% TC, 0.08–0.1% TN	Effect of Fe oxide and organic substrate addition on soil N transformations and *amoA* genes	Decrease of *amoA* abundance and GN and increase of NO_3^- immobilization is caused by high Fe oxide content rather than low pH	Jiang et al., 2015
nifH, amoA, hao, narG, nirK, nirS, norB, nosZ	GeoChip Microarray	Rain PTFs (China)	pH 4.4, 4.7–5.6% TC, 0.18–0.23% TN	Influence of environmental factors and chronic N deposition on functional gene diversity	High relative abundance of *amoA* and *narG* due to the high soil available N, which could be caused by the high rate of N deposition	Cong et al., 2015
nifH, amoA, hao, narG, nirK, nirS, norB, nosZ	GeoChip Microarray	PTF, STF and pasture in Amazonia (Brazil)	Sandy loam podzolic Latosol, pH 4.0–4.7, 1.43–2.02% TC, 0.1–0.15% TN	Influence of land use change on functional gene diversity, composition, and abundance	Nitrification genes are more abundant in forest sites than in pasture. *nifH, narG, nirS*, and *norB* were linked to both forest sites. Genes changes correlate with pH, Fe availability, texture, and organic matter	Paula et al., 2014
nifH	Clone libraries, qPCR	PTF, STF and pasture in Amazonia (Brazil)	Sandy loam podzolic Latosol, pH 4.6–5.8, 0.07–0.08% TC, 0.006–0.02% TN	Influence of land use change on free-living N-fixing microorganisms	*nifH* composition changes and its abundance increases following forest-to-pasture conversion due to changes in soil pH, TN, and C/N	Mirza et al., 2014
narG, nirK, norB, nosZ	Acetylene inhibition method (PD), qPCR	Conifer subtropical STF, shrub forest and farmland (China)	Acrisols and Ferralsols, pH 4.4–5.3, 0.95–2.47% TC, 0.08–0.22% TN	Influence of land use on denitrifiers abundance and total N gas production	PD, N gas production, and denitrifying genes were affected by land use change. PD, *norB*, and *nosZ* were positively correlated with pH	Yu et al., 2014
narG, nirK, norB, nosZ	Acetylene inhibition method (PD), qPCR, clone libraries	Temperate and subtropical forests (China)	Temperate: pH 4.5–7.4, Eh 650 mV; subtropical: pH 4.3–6.8, Eh 600 mV	The mechanisms governing low denitrification capacity and high N_2O emissions in subtropical forests soils	High Eh induced low denitrification capacity in subtropical soils (activity reduction of *nosZ*), being NO and N_2O the dominant gas products	Zhang et al., 2014
amoA	Shaken slurry method (PN), TRF profiles, clones libraries, qPCR	Seasonal evergreen TF and grassland (Trinidad)	Soils from different parent materials, pH 4.8–8.2, 0.39–2.98% TC, 0.06–0.35% TN	Influence of edaphic drivers on PN and nitrifying community structure	Soil N characteristics are significant for AOA, but not for AOB, and pH is not a major drive for AOA and AOB	de Gannes et al., 2014
amoA	Shaken slurry method (PN), clones libraries, qPCR	Humid TF (Puerto Rico, USA)	High weathered clay loam Ultisols, pH 3.9–5.4	Influence of oxic/anoxic fluctuation on PN and ammonia oxidizers	AOA community is tolerant to extended periods of anoxia. AOB were not detected	Pett-Ridge et al., 2013

(Continued)

TABLE 1 | Continued

N genes	Methods	Ecosystem	Edaphic characteristics	Condition tested	Major relationships in N functional genes	Reference
narG, nirK, nirS, nosZ	*In situ* measure of N_2O, acetylene inhibition method (PD), qPCR	Native wet sclerophyll forest (Australia)	Sandy Alfisol, pH 4.6–5.5, 3.5–6.9% TC; 0.10–0.33% TN	Effect of long-term repeated burning on N_2O flux, key soil properties, and denitrification gene abundance	More frequent fire reduced N_2O fluxes and C and N availability. Fire treatments did not significantly affect denitrification genes abundance	Liu et al., 2013b
amoA	^{15}N isotope technique (GN), clone libraries, culture, qPCR	Humid subtropical forests (China)	Sandstone Oxisol, pH 3.8–4.0, 2.6–4.6% TC, 0.1–0.19% TN	Influence of chronic N deposition on activity and composition of nitrifying community	Extraordinary abundance of AOA. AOB were not detected. Significant correlation between AOA abundance and GN rates.	Isobe et al., 2012
amoA	Shaken slurry method (PN), T-RFLP, clones libraries, qPCR	Tea orchard soils and pine subtropical forest (China)	Ultisol, pH 3.6–6.3, 0.003–0.071% TN	Long-term effects of low pH and N fertilization on the abundance, composition, and activity of AOA and AOB	AOA dominates in all sites and nitrification is driven by AOA in these acidic soils. Specific AOA and AOB populations occupy distinct pH niches.	Yao et al., 2011
nifH	Acetylene inhibition method (NFR), clone libraries, qPCR	Lowland rain TF (Costa Rica)	P-poor Ultisols	Links between N-fixer community structure from leaf litter, and changing P availability	P addition increased N fixation rates, N-fixers diversity and relative abundance, and the efficiency of N-fixers	Reed et al., 2010
amoA	Shaken slurry method (PN), clone libraries, PLFA	Atlantic lowland TF (Costa Rica)	Sandy loam soils, pH 5.2–5.8, 3.1–4.6% TC	Land-use types and plant diversity influence on AOB community	AOB differs among land-use types, but not across plant diversity, and correlates with PN	Carney et al., 2004

TF, tropical forest; PTF, Primary tropical forest; STF, Secondary tropical forest; NFR, N fixation rate; PN, potential nitrification; GN, gross nitrification; PD, potential denitrification; TC, total carbon; TN, total nitrogen; Eh, redox potential; DGGE, denaturant gradient gel electrophoresis; TRF, terminal restriction fragment; T-RFLP, terminal restriction fragment length polymorphism; qPCR, real-time PCR; AOA, ammonia- oxidizing archaea; AOB, ammonia- oxidizing bacteria; PLFA, phospholipid fatty acid composition, GHG, greenhouse gas.

currently available information regarding microbial functional groups that control the most commonly studied N processes (N fixation, nitrification, and denitrification), with an emphasis on unique aspects of their ecology in tropical forest soils.

Microbes Involved in N Fixation

Biological N fixation is the largest natural source of new N to most terrestrial ecosystems (Galloway et al., 2004). In this process symbiotic and free-living diazotrophic microorganisms reduce atmospheric dinitrogen gas (N_2) to reactive and biologically available forms. The *nifH* gene, encoding the reductase subunit of nitrogenase, the enzyme that catalyzes this reaction, has been widely used as a genetic marker to study the diversity and abundance of diazotrophs (Zehr et al., 2003; Gaby and Buckley, 2011). Likewise, a wide variety of PCR primer sets from multiple diazotrophic bacteria have been used to characterize and quantify the *nifH* gene in soils (Gaby and Buckley, 2012).

Relationships between diazotroph communities and N fixation rates have been investigated in many terrestrial ecosystems, including temperate forest soils (Rosch et al., 2002; Yeager et al., 2005), wetlands (Moseman et al., 2009), desert systems (Yeager et al., 2004; Wang et al., 2016), grasslands (Tu et al., 2016), and agricultural lands (Pereira e Silva et al., 2013). Despite the fact that tropical forest ecosystems maintain considerable biological N fixation to balance the large potential N losses, this process and the microbial community involved in it remain poorly investigated in these soils (Cusack et al., 2009; Reed et al., 2010; Mirza et al., 2014). Studies of lowland tropical rainforest in Costa Rica reported that soils were dominated by *nifH* clones related to the genera *Heliobacterium* (a member of the Firmicutes), as well as *Gluconacetobacter*, *Methylobacterium*, *Azospirillum*, and *Zymomonas* (all Alpha-proteobacteria; Reed et al., 2010). Diazotroph communities in primary and secondary rainforest soils in the western Amazon Basin of Brazil have been reported to be mainly composed of ubiquitous and abundant members of the Alpha and Beta-proteobacteria (e.g., the genera *Azospirillum*, *Azorhizobium*, *Bradyrhizobium*, *Methylobacterium*, *Burkholderia*), Firmicutes (e.g., *Paenibacillus*, *Heliobacterium*), and Cyanobacteria (e.g., *Nostoc*, *Anabaena*; Mirza et al., 2014). Interestingly, *nifH* sequences associated with methanogenic Archaea (*Methanoregula*, *Methanosphaerula*, *Methanocella*) were also found in these soils. Mirza et al. (2014) suggested that archaeal biological N fixation could play an important role in the Amazon rainforest.

Biological N fixation rates in tropical forests (15 to 36 kg N ha^{-1} yr^{-1}) are similar to or higher than estimates for their temperate counterparts (7 to 27 kg N ha^{-1} yr^{-1}), which are subjected to strong N limitation (Cleveland et al., 1999). Houlton et al. (2008) suggest that diazotrophs could be favored in tropical forests because they acquire enough N to maintain higher extracellular phosphatase activity, which is required to overcome P limitation in these ecosystems, and because the temperature

in tropical environments is near the optimum for biological N fixation. Furthermore, until recent years it was thought that free-living diazotrophs were the dominant form of N-fixing bacteria in temperate forest soils and symbiotic diazotrophs were the dominant form of N-fixing bacteria in tropical forest soils, where leguminous trees are common (Cleveland et al., 1999). However, recent studies in tropical forests indicate that free-living N-fixing bacteria in litter and soil may be more important than previously thought (Vitousek and Hobbie, 2000; Cusack et al., 2009; Hedin et al., 2009; Reed et al., 2013). Forest canopy communities, especially lichens, mosses, and other epiphytes associated with cyanobacteria, can also provide significant inputs of N to tropical forests (Matzek and Vitousek, 2003; Benner et al., 2007). Cusack et al. (2009) estimated N inputs via biological fixation by free-living microbes from above- and belowground components of two tropical forests in Puerto Rico, and assessed the response to increased N availability using an N fertilization experiment. Their results showed that free-living N fixation in soils was suppressed by N fertilization (50 kg N ha^{-1} yr^{-1}) and was significantly positively correlated with soil moisture, similar to findings in a tropical rainforest in Panama (Barron et al., 2008).

Tropical ecosystems are characterized by N fixation hotspots —zones where N fixation rates are much higher than average— that may reflect the very small-scale spatial heterogeneity of abiotic factors affecting the dynamics of the diazotroph community (Reed et al., 2010). While many tropical soils are generally nutrient-poor, there is a great deal of spatial and temporal heterogeneity in nutrient availability even on highly weathered soils (Townsend et al., 2008). The distribution and activity of N-fixing organisms in tropical forest soils are mainly regulated by moisture (Cusack et al., 2009), C/N ratio (Vitousek et al., 2002), and the availability of both P and molybdenum (Mo; Pearson and Vitousek, 2002; Benner et al., 2007; Reed et al., 2007; Barron et al., 2008; Wurzburger et al., 2012). Parent material can also drive the abundance of diazotroph communities in tropical soils. Stone et al. (2015) found that Inceptisol soils (primary-mineral rich soils) may provide a more favorable environment for diazotroph communities (and also denitrifier communities) compared with their Oxisol (acid soils, rich in Fe and Al secondary minerals) counterparts in two tropical forest types in Puerto Rico. Higher N loss rates due to leaching in Inceptisol subsoils could produce a feedback on surface N-cycle processes, increasing microbial N demand and ultimately stimulating N-fixation. In addition, anaerobic conditions can significantly increase rates of biological N fixation because O_2 inhibits the activity of the nitrogenase enzyme, and thus soil moisture content is an important factor in regulating rates of biological N fixation (Hicks et al., 2003).

Both P and micronutrients play important roles in regulating N fixation in tropical forests. The high P demand of diazotrophs to provide the large amounts of energy required by this process creates the potential for P limitation of N fixation. In addition, P in many lowland tropical forests is bound up with highly weathered soil minerals and organic matter. Microorganisms are able to liberate this P by exuding phosphatase enzymes and their synthesis requires large amounts of N. Thus, N fixation in tropical forests may confer a competitive advantage to diazotrophs by facilitating the uptake of P (Houlton et al., 2008). In line with these assertions, some experimental studies have shown that P addition stimulates this process (Benner et al., 2007; Reed et al., 2007), as well as increases the abundance and diversity of free-living diazotrophs in these ecosystems (Reed et al., 2010). Similarly, Mo is a critical component of the most common form of nitrogenase and some studies conducted in the tropical rainforest of Panama have suggested Mo limitation of N fixation (Barron et al., 2008; Wurzburger et al., 2012). Reed et al. (2013) performed a full-factorial litter incubation experiment to explore P and Mo controls over free-living N fixation rates from a Costa Rican tropical rainforest. Results showed that variation in P concentration (and not Mo) was positively correlated with N fixation rates. These authors asserted that more data are clearly needed to draw firm conclusions about general patterns in P vs Mo limitation of N fixation in these soils.

Nitrifying Microorganisms

Nitrification is the stepwise aerobic oxidation of NH_4^+ or ammonia (NH_3) to nitrite (NO_2^-) and NO_3^-. This key process plays an important role in regulating N availability and loss in terrestrial ecosystems, because it can cause NO_3^- leaching to groundwater and N_2O production directly (via chemical decomposition of hydroxylamine) and indirectly (via denitrification and nitrifier denitrification). Nitrification in soils is divided into autotrophic nitrification and heterotrophic nitrification. The former is mainly carried out by chemoautotrophic ammonium-oxidizing bacteria (AOB, represented by *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* species) and archaea (AOA, represented by *Nitrososphaera* and *Nitrosotalea* linages from the phylum Thaumarchaeota), and nitrite-oxidizing bacteria (NOB, represented by *Nitrobacter*, *Nitrospina*, *Nitrococcus*, and *Nitrospira* species). Heterotrophic nitrification is carried out by certain heterotrophic bacteria and fungi with the potential to oxidize both organic and inorganic N compounds (Hayatsu et al., 2008). Although it is widely accepted that autotrophic nitrification in soils is the prevalent nitrification process, high nitrification rates in tropical and subtropical acidic soils suggest that heterotrophic nitrification may be important in these soils (Pett-Ridge et al., 2013; Zhang et al., 2013), because it is less impacted by low pH than autotrophic nitrification (de Boer and Kowalchuk, 2001). In fact, using the ^{15}N tracing technique, both Zhang et al. (2011) and Zhu et al. (2013) found that heterotrophic nitrification was the dominant process producing NO_3^- in subtropical acidic coniferous forest, where autotrophic nitrification rate was very low. Using the ^{15}N tracing method and a selective biomass inhibitor (fungal inhibitor: cycloheximide and bacterial inhibitor: streptomycin), Zhu et al. (2015) identified that fungal rather than bacterial pathways dominate heterotrophic nitrification in the same forest soils, suggesting that fungi may be the important driver for heterotrophic nitrification in acid forest soils of subtropical China. However, the ecology of heterotrophic nitrifier microorganisms has not yet been investigated in tropical forest soils.

Nitrifier denitrification is an alternative pathway and involves the oxidation of NH_3 to NO_2^- and its subsequent reduction via NO to N_2O by AOB (Colliver and Stephenson, 2000), particularly

by *Nitrosospira* taxa (Shaw et al., 2006). Recent studies have suggested that this process may contribute considerably to N_2O fluxes from soils with high N content and low pH and oxygen pressure (Wrage et al., 2004; Kool et al., 2010; Gharahi Ghehi et al., 2012). These conditions are typical in tropical forest soils, thus we speculate that nitrifier denitrification could play an important role in these ecosystems. However, to our knowledge, no studies directly related to this process have been conducted in tropical forests soils.

Microbial ammonia oxidation is the first and rate- limiting step of autotrophic nitrification, which may explain why the few studies on microbial nitrification in tropical forest soils have mainly focused on this process. Ammonia-oxidizing microorganisms oxidize NH_3 to NO_2^- by using the ammonia monooxygenase enzyme, the α-subunit of which is encoded by the *amoA* gene, a common genetic marker for nitrification (Rotthauwe et al., 1997; Treusch et al., 2005). It was presumed that chemolithotrophic Gamma- and Betaproteobacteria were primarily responsible for this process in soils (Kowalchuk and Stephen, 2001). However, recent studies have demonstrated that AOA are ubiquitous constituents of terrestrial environments (Leininger et al., 2006; Pester et al., 2012; Zhalnina et al., 2012; de Gannes et al., 2014) and their discovery has changed the paradigm of aerobic nitrification. The ecology of AOA remains under investigation, and there is not a simple relationship between NH_3 oxidation rates and the relative abundances of AOA and AOB (Nicol and Schleper, 2006; Taylor et al., 2012).

In soils, drivers of niche differentiation of AOA vs AOB communities have centered largely on pH, NH_3 concentrations and N organic forms. High abundances of archaeal *amoA* genes have been reported in many acidic soils (Leininger et al., 2006; Boyle-Yarwood et al., 2008; Chen et al., 2008; Stopnišek et al., 2010; Isobe et al., 2012) and multiple studies suggest that some groups of AOA may prefer a pH below 5.5 (Nicol et al., 2008; Gubry-Rangin et al., 2010; Yao et al., 2013). In strongly acidic soils, AOA abundance increases or remains unchanged with decreasing pH, while AOB abundance decreases (He et al., 2007; Yao et al., 2011; Taylor et al., 2012). However, in neutral to alkaline soils, correlations of AOA or AOB abundance to pH have not been consistent (Shen et al., 2008; Jia and Conrad, 2009). Investigations have also indicated a preference of soil AOA communities for low NH_3 levels (Wessén et al., 2010; Verhamme et al., 2011) and low organic nutrient conditions (Erguder et al., 2009).

Database surveys focused on soils from temperate regions have revealed a strong biogeographical component to the distribution of AOA and AOB phylotypes (Fierer et al., 2009a; Pester et al., 2012). However, in tropical soils very little is known about ammonia-oxidizing microorganisms and how environmental factors affect their activity. Many tropical forest soils tend to be wetter, have smaller annual fluctuations in temperature, lower pH, high available N and more reduced conditions than temperate forest soils, and this may impose different environmental conditions (and require different traits) for ammonia-oxidizing organisms. For instance, *Nitrosospira* cluster 2 has been found in great abundance in acidic soils (Boyle-Yarwood et al., 2008), but it has been suggested that it may be more common in cold-temperate forest soils than in warmer tropical soils (Avrahami and Conrad, 2005; Fierer et al., 2009a). So far, ammonia oxidizer *amoA* sequences from clone libraries have been described from only a few wet tropical forest soils from Trinidad (de Gannes et al., 2014), Puerto Rico (Pett-Ridge et al., 2013), and Costa Rica (Carney et al., 2004), as well as from two different subtropical forest soils in China (Yao et al., 2011; Isobe et al., 2012). Only three of these studies have successfully amplified bacterial *amoA* sequences from these soils, with *Nitrosospira* and *Nitrosomonas* the primary taxa (Carney et al., 2004; Yao et al., 2011; de Gannes et al., 2014). The majority of archaeal *amoA* sequences described in these soils clustered with sequences from *Nitrososphaera* and *Nitrosotalea* (Pester et al., 2012; Pett-Ridge et al., 2013; de Gannes et al., 2014).

Similar to many acidic temperate forest soils, AOA populations have been inferred to be the primary group driving nitrification in tropical forest soils (Yao et al., 2011; Lammel et al., 2015). Furthermore, Isobe et al. (2012) and Pett-Ridge et al. (2013) were unable to amplify bacterial *amoA* genes from acidic tropical forest soils (Supplementary Table S2). At least two other studies examining acidic forest soil have found a similar pattern of absence of bacterial *amoA* genes (Stopnišek et al., 2010; Levičnik-Höfferle et al., 2012). Thus, one possible explanation for the dominance of AOA in these soils is the relatively low soil pH (Nicol et al., 2008; Gubry-Rangin et al., 2010; Yao et al., 2013). For example, Yao et al. (2011) observed that the ratio of AOA to AOB *amoA* gene abundance increased with decreasing soil pH in subtropical soils in China. Significant relationship between AOA abundance and nitrification potential was also found, indicating that nitrification was mainly driven by AOA in these acidic soils. However, de Gannes et al. (2014) found that AOA abundance predominated over AOB in tropical soils that spanned pHs of 4 to 8 in Trinidad (Supplementary Table S2), and the community abundance and structure of AOA was affected primarily by soil N characteristics, but not by soil pH. According to these authors, the effect of pH on nitrification appeared to mainly reflect impacts on AOA or AOB activity, rather than selection for AOA or AOB phylotypes differing in nitrifying capacity. In addition, Jiang et al. (2015) found higher net nitrification rate and abundance of AOA and AOB for Cambisols than for Ferralsols from the same parent material in a subtropical acidic forest in China (Supplementary Table S2). These authors also reported higher NO_3^--N immobilization for Ferralsols than for Cambisols, which imply that the lower nitrification rate and *amoA* abundance in these Ferralsols may due to high amounts of NO_3^--N immobilization stimulated by high Fe oxide concentrations. A potential mechanism of abiotic immobilization of NO_3 has been postulated, suggesting that Fe plays a key role in NO_3^--N immobilization by promoting organic N formation from inorganic N (Davidson et al., 2003). Thus, *amoA* genes abundance may be more affected by soil nutrients and Fe oxide contents rather than a low pH in tropical forest soils.

In contrast to temperate forest soils, tropical soils routinely experience fluctuating O_2 availability over short time scales. This could represent a unique selective habitat for ammonia oxidizers adapted to this O_2 regime. Laboratory measurements in Puerto

Rican forest soils showed elevated NH_4^+ pools and high gross nitrification rates immediately following periods of anaerobiosis, indicating that substantial NH_4^+ oxidation occurs despite low pH and limited O_2 availability (Pett-Ridge et al., 2006, 2013). The survival mechanisms of these nitrifiers during periods of prolonged anaerobiosis are not well known. It has been shown that under oxygen-limiting conditions, ammonia oxidizers can use NO_2^- as a terminal electron acceptor (Shrestha et al., 2002), or exploit the oxic-anoxic spatial segregation (Sey et al., 2008). Another explanation is that NH_4^+ is constantly regenerated via DNRA due to the redox fluctuation conditions in tropical soils (Silver et al., 2001; Templer et al., 2008), and with a subsequent influx of O_2, this NH_4^+ becomes reavailable to ammonia oxidizer communities. On the other hand, AOA populations appear to be insensitive to O_2 availability (Zhalnina et al., 2012). Tropical soil AOA not only tolerate extended periods of low O_2 (Pett-Ridge et al., 2013), but can react faster than AOB in alternating aerobic/anaerobic conditions (Chen et al., 2008). It has also been proposed that the pathway of NH_4^+ oxidation in AOA may be distinct from the AOB pathway, theoretically requiring only 0.5 O_2 per NH_4^+ oxidized (Walker et al., 2010). This hypothesized lower O_2 demand could explain why AOA populations seem to persist more readily under low-oxygen conditions.

A lack of correspondence between nitrification rates and nitrifier community abundance in tropical forest soils has been reported by some investigators (Pett-Ridge et al., 2013; de Gannes et al., 2014), and the reasons for this disconnection are unknown. It is possible that unique ammonia-oxidizers exist in these ecosystems, which can evade detection by the PCR primers developed from currently available sequence data. It is tempting to speculate that the particular conditions of these soils may select for specific lineages of ammonia oxidizers, although much more molecular microbial diversity data from these soils and additional characterization of soil isolates are needed to test this hypothesis.

Denitrifying Microorganisms

Denitrification is a microbial anaerobic respiration pathway through which NO_3^- or NO_2^- are sequentially reduced to N_2 via the intermediates NO and N_2O. It is the major biological process in soils that returns fixed N to the atmosphere and closes the N cycle (Philippot et al., 2007). It is also the primary pathway of NO and N_2O emissions from soil (Houlton and Bai, 2009). Denitrification consists of four reactions catalyzed by the metallo-enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Zumft, 1997). Nitrite reductase is the key enzyme of denitrification, since it catalyzes the reduction of NO_2^- to gas products (Henry et al., 2004), which is the reaction that limits the rate of the denitrification process (Zumft, 1997). Several studies have used genetic markers for denitrification, including *narG* and *napA* (markers for nitrate reductase), *nirK* and *nirS* (markers for nitrite reductase), *norB* (a marker for nitric oxide reductase) and *nosZ* (a marker for nitrous oxide reductase), to study the diversity of denitrifying microorganisms in various environments (Henry et al., 2006; Kandeler et al., 2006; Smith et al., 2007; Yu et al., 2014). Denitrifier organisms can have different combinations of genes involved in the denitrification pathway (Zumft and Kroneck, 2006; Jones et al., 2008). Likewise, while some microorganisms harbor all denitrification enzymes and can potentially perform the complete pathway, others lack the nitrous oxide reductase gene and produce only N_2O as the denitrification end product (Philippot et al., 2011). Furthermore, recent studies have identified non-denitrifying N_2O reducers with atypical *nosZ*, which are potential contributors to N_2O reduction in soils (Sanford et al., 2012; Jones et al., 2013). Thus, understanding the ecology of this functional guild is potentially important for mitigating N_2O emissions from soils. This newly identified clade of N_2O reducers is diverse and widespread in terrestrial environments (Jones et al., 2013), but the ecology of this group in tropical forest soils is still unknown.

Denitrification is distributed among a taxonomically diverse group of microorganisms, which have acquired the ability to denitrify via different evolutionary mechanisms (Tiedje et al., 1983; Jones et al., 2008). This process in soil is mainly performed by facultative aerobic heterotrophic bacteria, such as some species of *Pseudomonas*, *Bacillus* and *Paraccocus*, and autotrophic bacteria, such as *Thiobacillus denitrificans* (Philippot et al., 2007; Demanèche et al., 2009). Denitrification is also found among a few archaea (Cabello et al., 2004; Bartossek et al., 2010) and fungi (Shoun et al., 1992; Hayatsu et al., 2008), including Ascomycota (e.g., *Fusarium oxysporum*, *Fusarium solani*, *Cylindrocarpon tonkinense* and *Gibberella fujiuroii*) and Basidiomycota (e.g., *Trichosporon cutaneum*). Fungi generally lack N_2O reductase (Shoun et al., 1992), suggesting that these organisms are responsible for a large portion of N_2O emission from soil. Despite the frequent observation that tropical microbial communities in soil are often dominated by fungi (Hawksworth, 2012), their contributions to N_2O emissions from tropical forest soils have not yet been addressed. The polyphyletic distribution of denitrifying genes can result in their co-occurrence with N-fixation and ammonia-oxidation genes in many microbes (Bedmar et al., 2005; Hayatsu et al., 2008). Many diazotrophic bacteria, such as some species of *Azospirillum* and *Bradyrhizobium*, can denitrify (Rosch et al., 2002). Similarly, AOB belonging to either the genus *Nitrosospira* or *Nitrosomonas* have been shown to be capable of denitrification (Shaw et al., 2006).

Understanding the link between ecosystem scale denitrification rates and the ecology of the microorganisms that mediate this pathway has been identified as a critical research goal for the mitigation of climate change (Richardson et al., 2009). For this reason, numerous studies have been conducted on the microbially mediated denitrification process in temperate soils, especially its role in N losses and its sensitivity to various environmental factors (Hofstra and Bouwman, 2005; Katsuyama et al., 2008; Attard et al., 2011; Dandie et al., 2011). Despite the growing evidence that denitrification mechanisms and denitrifier communities in tropical forest soils may be very different from those of temperate soils (due to their unique soil characteristics and environmental conditions; Zhang et al., 2009; Xu et al., 2013), few studies exist that document the relationship between denitrifier abundance and total N gas production in tropical and subtropical soils (Liu et al., 2013b; Yu et al., 2014; Zhang et al., 2014; Lammel et al., 2015; **Table 1**; Supplementary Table S2). Furthermore, contradictory results have been published about

the importance of denitrification as a significant route of N loss in tropical ecosystems. Some studies have shown that tropical forest soils are characterized by generally lower denitrification capacity than temperate forest soils, with greater variability both temporally and spatially due to heterogeneity in edaphic conditions (Robertson and Tiedje, 1988; Xu and Cai, 2007; Zhang et al., 2014). However, tropical soils are considered to be the largest natural sources of NO and N_2O due to the combination of climatic factors, soil properties, and rapid rates of N cycling (Davidson et al., 2001; Neill et al., 2005; Stehfest and Bouwman, 2006). The conceptual "hole-in-the-pipe" model gives a simplified view of the main controls on N_2O and NO fluxes (Firestone and Davidson, 1989). In this model, the rate of nitrification and denitrification is conceptualized as a pipe, while the relative ratio of NO and N_2O to total gas produced is conceptualized as a hole. According to Zhang et al. (2014), N flowing "through the pipe" is significantly less in subtropical forest soils than in temperate forest soils. However, the "holes in the pipe" are much bigger in subtropical forest soils than in temperate forest soils, resulting in a larger amount of NO and N_2O gases "leaking out of the pipe". High redox potential (resulting from enrichment of iron and aluminum oxides), low pH and high N availability are the key factors affecting the amount of N flowing through the pipe and the size of the holes in the pipe. These factors may suppress complete denitrification in humid tropical soils and enhance NO and N_2O ratios during denitrification (Zhang et al., 2009).

The quantification of denitrification genes to elucidate the relative importance of abiotic environmental factors versus denitrifier distribution has been recently achieved in tropical forest ecosystems (Paula et al., 2014; Yu et al., 2014; Zhang et al., 2014; Lammel et al., 2015; Stone et al., 2015; **Table 1**; Supplementary Table S2). These studies indicate that many soil properties can affect denitrifier community attributes and denitrification rates in tropical soils, including parent material, pH, redox potential, O_2 partial pressure, available organic C and N, and NO_3^- concentration. For example, the abundances of denitrification genes (*nirS* and *nosZ*) were positively correlated with soil C, N, and P concentrations and were more abundant in the Inceptisol compared with the Oxisol soil type in humid tropical forests in Puerto Rico (Stone et al., 2015), suggesting more dynamic N transformation processes in the sandier soil sitting on a younger, more rapidly weathering parent material. Yu et al. (2014) found that the abundance of both *norB* and *nosZ* gene copies were positively correlated with soil pH, and NO and N_2O fluxes from acidic forest soils (pH 4.4−4.7) were lower than in farmland soils with higher pH (5.0−5.3) in a subtropical region of China. On the other hand, Zhang et al. (2014) showed that redox potential, rather than soil pH or organic C, was the key soil variable influencing denitrification capacity and the abundance of denitrifiers in subtropical forest soils from China. Plant species composition has also been suggested as an important factor in determining the distribution and abundance of denitrifiers in soil (Bremer et al., 2007). So far, only one study has addressed the influence of plant species composition on the abundance of soil N functional genes in tropical soils (Reverchon et al., 2015). These authors found that inter-planting teak with fluegge increased N stocks and the relative abundance of denitrification genes compared to teak mono-plantations in the Salomon Islands, suggesting that mixed species plantations may potentially alleviate N losses and favor N retention.

Lammel et al. (2015) found that pristine rainforest soils in Amazonia showed higher N content and abundances of denitrifier genes (*nirK* and *nosZ*) than pasture and crop fields, which correlated with N_2O emissions. However, Liu et al. (2013b) found no correlation between denitrifier abundance and N_2O fluxes in a tropical forest soil in Australia, suggesting that soil C and N substrate availability and soil environmental factors (pH and moisture) rather than denitrification gene abundance control N_2O fluxes in this ecosystem. Studies in other soil environments have also reported that changes in denitrification rate were not associated with changes in denitrifier community structure (Hallin et al., 2009; Attard et al., 2011). This may be attributed to the high functional redundancy commonly found in phylogentically diverse groups such as denitrifying bacteria. A further understanding of this topic requires research on the expression of denitrification genes (e.g., via RNA-based approaches that target physiologically active populations in denitrification) and its relation to denitrification rates, as well as to separate N_2O produced through denitrification from nitrifier denitrification.

It is well known that O_2 (Bollmann and Conrad, 1998), organic C and NO_3^- availability (Klemedtsson et al., 2005), low pH (Šimek et al., 2002), or sudden onset of anoxia (Morley et al., 2008) can also influence denitrification rates and negatively affect the reduction of N_2O to N_2. However, the mechanisms that increase gene transcription and enhance N_2O reductase activity have not been researched in detail in tropical forest soils. For example, soil pH is one of the main factors controlling broad-scale microbial activity and the stoichiometry of denitrification (Šimek et al., 2002; Liu et al., 2010). This process in acid soils, such as typical tropical forest soils, seems to be particularly disposed to high N_2O/N_2 ratios (Šimek et al., 2002; Zhu et al., 2013), apparently due to impaired functioning of N_2O reductase at low pH (Liu et al., 2010). However, contradictory results have been published on the relationship between soil pH and denitrification rates in tropical and subtropical acid soils in China. Xu and Cai (2007) showed that denitrification capacity was not correlated with soil pH in humid subtropical soils, but with organic carbon content. Furthermore, no correlation between soil pH and denitrification rate was found in six forest soil types in different climatic zones (temperate, tropical and subtropical forests; Zhang et al., 2009). On the other hand, Yu et al. (2014) reported that low soil pH (<4.7) decreased denitrification rates and NO and N_2O emissions in subtropical forest soils from China.

Soil moisture is a major driver of N_2O emissions as it regulates the oxygen availability to soil microbes. N_2O emissions have their optimum in the range of 70–80% water-filled pore space depending on soil type (Davidson et al., 2000). Since denitrification occurs as soil O_2 becomes limiting, denitrifiers are likely to be more widespread and resilient in tropical soils due to their temporally and spatially variable redox environment (Pett-Ridge et al., 2006). These taxonomically and physiologically diverse facultative microorganisms preferentially respire O_2, but can reduce NO_3^- and other partially oxidized N-forms when O_2

is limiting (Tiedje et al., 1983; Tiedje, 1988). Pett-Ridge et al. (2006) performed a fluctuating redox incubation experiment using tropical forest soils from Puerto Rico and found that denitrification tended to be highest in soils that had been anoxic for several weeks. These results indicated that denitrifiers might dominate in situations where O_2 is consistently limiting, whereas microbes that mediate DNRA may be more competitive for NO_3^- in a fluctuating redox environment (Tiedje, 1988). On the other hand, frequent oxic/anoxic transitions may also inhibit the expression of nitrous oxide reductase, since its activity is more sensitive to O_2 compared with other denitrifying enzymes (Morley et al., 2008).

ANTHROPOGENIC FACTORS INFLUENCING MICROBIAL N CYCLING IN TROPICAL FOREST SOILS

Alteration of the N cycle by human activities affects climate, food and energy security, human health, and ecosystem services (Erisman et al., 2013). Anthropogenic factors also have a great potential to alter N processes in tropical forests and significant changes in N dynamics in these forests is expected, including rising NO_3^- export to ground and surface waters, threatening drinking water quality and increasing eutrophication risks (Matson et al., 1999; Corre et al., 2010). Furthermore, in the coming decades, the tropics will experience unprecedented increases in temperature and significant alterations in precipitation regime, which also have the potential to affect the N cycle (Diffenbaugh and Scherer, 2011; Bai et al., 2013; IPCC, 2013). For example, soil warming may increase microbial metabolism and enzyme activity, resulting in greater organic matter decomposition and gross N mineralization (Koch et al., 2007). Warmer temperatures could also stimulate additional N losses via increased denitrification rates (Bai et al., 2013). However, so far very little is known regarding the response of microbial communities involved in N processes to different components of global environmental change in the tropics. It is beyond the scope of this paper to review the myriad microbial responses to environmental change; rather, we focus our review on the limited currently available research regarding the response of microbial N cycling to anthropogenic factors in tropical forest soils, such as chronic-elevated N deposition, forest perturbation, and land-use change.

N deposition

Atmospheric deposition of reactive N compounds has increased threefold during the last century due to fossil fuel combustion, artificial fertilizer application and leguminous crops (Vitousek et al., 1997; Gruber and Galloway, 2008). Currently, N deposition adds more than 200 Tg yr^{-1} of N to terrestrial and ocean ecosystems, and is predicted to increase between 50 and 100% by 2030 compared with 2000 (Gruber and Galloway, 2008; Reay et al., 2008). Furthermore, reactive N deposition is dramatically increasing in the tropics because of economic development and increasing biomass burning (Galloway et al., 2004; Hietz et al., 2011), resulting in many unexpectedly negative effects. In fact, elevated N deposition not only occurs in areas with high population densities but also in areas far from the source of N. For example, southern Ecuadorian montane forests receive elevated atmospheric N and P deposition that is largely attributed to biomass burning in the Amazon basin (Fabian et al., 2005). In addition, recent climate models that incorporate N deposition as a part of the CN-coupled models predict a lower net C uptake (37–74%) than values projected by models using C cycle components alone, demonstrating the importance of the N cycle in future climate change (Thornton et al., 2009). Thus, a priority in terrestrial ecosystem research is to determine the fate of N, its interactions with soil microbial communities and its coupling with other biogeochemical cycles, such as the C cycle (Bardgett et al., 2008; Reay et al., 2008; Singh et al., 2010).

The responses of soil N transformations to N addition depend on forest N status, soil characteristics, and climatic factors (Lohse and Matson, 2005; Martinson et al., 2012; Corre et al., 2014). Anthropogenic N deposition has been shown to greatly affect N cycling in temperate and boreal forests, where low availability of N limits primary productivity. These responses have been well studied and summarized in the conceptual model known as the "N saturation hypothesis" (Aber et al., 1998; Galloway et al., 2003). The concept of "N saturation" refers to a state in which N supply exceeds biological demand, resulting in the loss of new N from the ecosystem by biological or physical mechanisms. In this model, atmospheric chronic N deposition in temperate and boreal forests increases soil acidification, stimulates N loss through extensive NO_3^- leaching and gaseous N emissions, and produces a switch from N to P limitation and a decline in plant diversity (Aber et al., 1998; Vitousek et al., 1997, 2010). In contrast to temperate forests, tropical forests are generally considered N-rich relative to other nutrients, and thus N deposition is less likely to increase primary productivity of tropical ecosystems, but may alter other aspects of the N cycle (Corre et al., 2010; Hietz et al., 2011). It has been also hypothesized that chronic N deposition will acidify tropical forest soils, further exacerbate P deficiency in highly weathered soils, reduce tree growth and C storage, and negatively affect biodiversity (Matson et al., 1999; Vitousek et al., 2010). Recent evidence suggests that these changes are currently occurring in tropical forests (Hietz et al., 2011; Lu et al., 2014).

Compared with other ecosystem types, tropical forests emitted more N_2O under N enrichment (on average +739%; Liu and Greaver, 2009), which suggests strong feedbacks of soil N_2O emissions on increasing atmospheric N deposition in the tropics. However, research on the effect of N deposition in tropical and subtropical forest ecosystems is much more recent, and the temporal scale of such studies much shorter than those in temperate forests. For example, the effect of N deposition on N-saturated tropical forest ecosystems in southern China has been recently studied. Forests in this region have acidified soils, exhibit chronic atmospheric N deposition of 16−38 $kg{\cdot}ha^{-1}{\cdot}yr^{-1}$ (Chen and Mulder, 2007; Fang et al., 2011) and high throughfall N input that vary greatly from 11.7 to 65.1 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$ (Fang et al., 2011). It had been shown that N_2O emission significantly increases, but soil pH, primary production, litter decomposition and

N mineralization significantly decreases in high N addition (100−150 kg N·ha^{-1}·yr^{-1}) plots (Lu et al., 2009; Zhu et al., 2013; Wang et al., 2014). These results are consistent with progressive P limitation, which results in higher litter N content and soil N availability. Likewise, a two-year N addition experiment (50−150 kg N·ha^{-1}·yr^{-1}) was performed in a humid subtropical bamboo forest in China, which exhibits one of the highest chronic atmospheric N deposition rates in the world (113.8 kg N·ha^{-1}·yr^{-1} in 2009; Tu et al., 2013). In this ecosystem, N deposition accelerated nitrification and denitrification rates, increased hydrolytic enzyme activities, soil N availability and microbial biomass, and decreased soil P availability (Tu et al., 2014). Results suggest that this bamboo forest ecosystem is moving toward P limitation under elevated N deposition, and may increase N_2O emission and NO_3^- leaching.

The same pattern of increased N-oxide emissions and NO_3^- leaching induced by N deposition has been found for montane tropical forests. In Hawaiian montane forests, 11−13 years of high N addition (100 kg N·ha^{-1}·yr^{-1}) to an N-limited forest on an Andosol soil and 5−7 years of high N addition (100 kg N·ha^{-1}·yr^{-1}) to a P-limited forest on a Ferralsol soil increased gross rates of mineral N production but decreased microbial N retention, leading to higher soil N-oxide emissions and NO_3^- leaching (Hall and Matson, 2003; Lohse and Matson, 2005). Similarly, 1−4 years of high N addition (125 kg N·ha^{-1}·yr^{-1}) in an N-limited Panamanian montane forest on an Andosol soil increased gross rates of soil N mineralization and nitrification, leading to increases in soil N-oxide emissions and NO_3^- leaching (Corre et al., 2010, 2014). Corre et al. (2014) also showed that this wet montane forest with only 4 years of N addition had comparable N-oxide emissions to an N-rich moist lowland forest in Panama with more than a decade of N addition.

Elevated N deposition in tropical forests may also significantly change soil microbial community composition and activity (Liu et al., 2013a; Tu et al., 2014). There are two primary hypotheses that seek to explain the influence of N addition on microbial community dynamics. The N-mining hypothesis proposes that under N enrichment, microbes reduce decomposition of recalcitrant C in response to their lowered N requirements, resulting in a shift toward labile C decomposition and reduced overall microbial activity (Chen et al., 2014). The copiotrophic hypothesis suggests that N additions decreases the relative abundance of oligotrophic taxa (k-strategists, i.e., slow growing microbes that have high substrate affinity and can catabolize more recalcitrant C pools), which would be out-competed by more copiotrophic taxa (r-strategists, i.e., fast growing microbes that have high nutritional requirements and preferentially consume labile soil organic C pools; Fierer et al., 2007, 2012a; Ramirez et al., 2012).

Knowledge about the effects of N deposition on particular microbially mediated N processes in tropical forests is still limited and controversial (**Table 1**). Cusack et al. (2009) reported that N fertilization (50 kg N ha^{-1} yr^{-1}, which is approximately twice the average projected rate for Central America for the year 2050; Galloway et al., 2004) suppressed soil biological N fixation in two tropical forests in Puerto Rico, similar to findings in a rain tropical forest in Panama (Barron et al., 2008). Isobe et al. (2012) investigated nitrifying communities of acidified subtropical forests soils in southern China and found that archaea rather than bacteria control NH_4^+ oxidation after long-term N deposition (32−34 kg N·ha^{-1}·yr^{-1}). However, it has been reported that rates of heterotrophic nitrification were significantly higher than those of autotrophic nitrification in these forest soils (Zhang et al., 2013). Cong et al. (2015) found that the high relative abundance of both *amoA* and denitrification genes, especially *narG*, in a tropical rainforest soil in China was due to the large amount of soil available N (from 141.4 to 224.5 mg kg^{-1}), caused by the high rate of N deposition (over 25 kg N·ha^{-1}·yr^{-1}) that affects this ecosystem. In sum, there is evidence indicating that N deposition in tropical forest soils may inhibit biological N fixation (Barron et al., 2008; Cusack et al., 2009), alter N mineralization and nitrification (Zhao et al., 2007; Corre et al., 2010), and induce more N_2O emissions and NO_3^- leaching, particularly during the wet season (Hall and Matson, 1999; Corre et al., 2014; Wang et al., 2014). However,

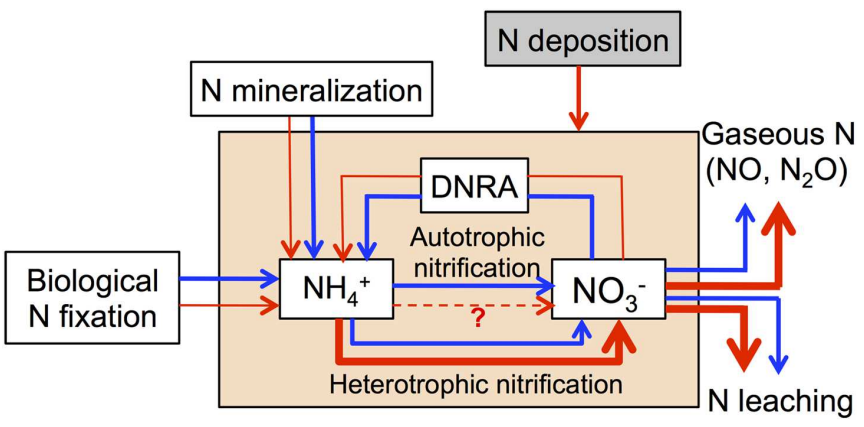

FIGURE 2 | Conceptual model of how chronic-elevated N deposition affects microbially mediated N processes in N-rich tropical forests soils. Blue arrows represent N processes without N deposition and red arrows represent N processes with N deposition.

the consequences of these changes at the ecosystem scale are not well understood.

We summarize all available evidence in a conceptual model of how increasing N deposition may affect microbially mediated N processes in N-rich tropical forests soils (**Figure 2**). We hypothesize that chronic-elevated N deposition may lead to a decrease of biological N fixation, N mineralization and DNRA, as well as an increase of heterotrophic nitrification and denitrification, which will lead to high N losses via N leaching and gaseous N production. Furthermore, we also emphasize that these processes are affected by multiple factors such as the initial soil N status, soil type, soil moisture and temperature, elevation (montane or lowland), and landscape position (Martinson et al., 2012; Zhu et al., 2013; Corre et al., 2014; Stone et al., 2015), which may alter the responses of these processes to N deposition.

Land-Used Change and Forest Perturbation

Land use change is considered the most important factor driving biodiversity losses in tropical areas (Sala et al., 2000). In recent decades, tropical forests have undergone rapid conversion to agriculture, with 80 million hectares of forest cleared in the 1980s and 1990s (Gibbs et al., 2010). Logging and forest conversion result in a series of ecosystem alterations that include an increase in belowground productivity (Cenciani et al., 2009), changes in soil chemistry (McGrath et al., 2001; Murty et al., 2002), and a more open N cycle (Davidson et al., 2007). Following deforestation, not only plant biomass but also the soil stocks of total C and N can become mobilized and be emitted in the form of greenhouse gases (Neill et al., 2005; Galford et al., 2010; Arai et al., 2014). Among these emissions, N_2O has a global warming potential for 100 years 265-298 times greater than that of the equivalent amount of CO_2 (IPCC, 2013) and promotes ozone destruction in the stratosphere. Thus, understanding the controlling factors of N_2O emission is crucial for quantifying the effects of land-use change on the N cycle and for managing and mitigating the environmental consequences associated with N pollution (Galloway et al., 2004).

It has been shown that land use change in the tropics also alters soil bacterial (da Jesus et al., 2009; Rodrigues et al., 2013; Ranjan et al., 2015), fungal (Mueller et al., 2014), and archaeal (Taketani and Tsai, 2010) community composition. For example, a recent study showed that the conversion of Amazonian forest to pasture impacts microorganisms, resulting in biotic homogenization of communities (Rodrigues et al., 2013). Various studies have suggested that deforestation of tropical forests for agriculture and agroforestry decreases microbial biomass and alters the soil microbial composition (Bossio et al., 2005; Arai et al., 2014; Krashevska et al., 2015). However, the impact of land use changes on soil N processes and the microorganisms responsible for these processes has been limited to only a few studies (e.g., Carney et al., 2004; Yu et al., 2014; Lammel et al., 2015; **Table 1**; Supplementary Table S2).

So far, only one study has integrated measures of greenhouse gas emissions and the abundance of multiple functional and phylogenetic genetic markers (*nifH, amoA, nirK, nirS, norB, nosZ, mcrA, pmoA*, and *16S/18S rRNA*) with environmental parameters to predict the response of biogeochemical processes to land use change in Amazonian soils (Lammel et al., 2015). Results showed that land use change from forest to agriculture reduced the abundance of different functional microbial groups related to C and N cycles in the wet season, but its effects were less significant after the dry winter fallow. Paula et al. (2014) studied the influence of land use change from forest to pasture on functional gene diversity, composition and abundance in Amazon rainforest soil microbial communities. They found that genes linked to C and N cycling were the most altered by deforestation. Carney et al. (2004) also observed that soil AOB diversity and composition changed significantly with land use in the tropical lowland of Costa Rica, and these changes were significantly associated with shifts in the potential rates of nitrification. The work of Mirza et al. (2014) is one of the first studies using the marker gene *nifH* to assess the response of free-living diazotrophs to deforestation in the Amazon rainforest. They reported a shift in the diazotrophic community composition and an increase in *nifH* gene abundance following forest-to-pasture conversion. They suggested that the response of diazotrophs to land use change is a consequence of changes in plant communities, particularly the higher N demand of pasture for supporting aboveground plant growth. Yu et al. (2014) also found that the abundance of denitrifying genes (*narG, nirK, norB*, and *nosZ*) was significantly affected by land-use change in subtropical soils of China.

CONCLUSION AND PERSPECTIVES

Most studies of the consequences of N cycle alteration induced by anthropogenic environmental changes have been performed in temperate forests where biological processes are limited by N supply. However, the response of N processes

BOX 1 | Future research on microbial ecology of the N cycle in tropical forest soils.

- The contributions of different microbial subpopulations to the N cycle.
- The ecology of recently discovered processes, such as anammox and codenitrification.
- Modeling microbial regulation of the N cycle.
- Network analyses to explore links between environmental factors and microbial control of the N cycle.
- Links between specific microbial metabolic processes and the C and N cycles.
- The interactive effects of C, N, and P on the soil microbial community.
- The influence of precipitation and soil warming on the community structure of N-cycling microorganisms.
- The influence of soil spatial heterogeneity on microbial community functions and N cycling regulation.
- The impact of disturbance on microbial community structure and function and its consequences for the N processes at global scales.
- Microbial community responses to multiple experimental climate change drivers.
- Mechanisms regulating the activity and interaction between primary producers and microorganisms controlling N cycling (plant–microbial feedbacks for the regulation of N).

to environmental change in tropical forest soils is largely unknown. In order to better understand, predict and mitigate global environmental changes, there is an urgent need to clearly understand the microbial control of greenhouse gas emissions and its interactions with environmental factors. Furthermore, a deeper understanding of microbially mediated N processes in tropical forest soils is crucial as N cycling in these soils impacts food production for a large part of the world, as well as the dynamics of global climate change.

This review reveals large knowledge gaps regarding microbially mediated N processes in tropical forest soils, gaps that represent critical directions for future research. We propose several topics of research that should be prioritized (**Box 1**). For example, to more accurately predict the response of N cycling to environmental change in tropical forest ecosystems, additional data regarding how N cycling is regulated is needed from a much broader range of tropical forests than currently studied, as well as improvements in our ability to quantify *in situ* the rates of the primary N processes in these soils. Likewise, it is important to carry out studies in tropical forest soils of recently discovered processes, such as anammox and codenitrification. In addition, research is required to link these processes with soil microbial community characteristics and the functional roles of microbial extracellular enzymes, in order to provide mechanistic insights into microbial regulation of the N cycle in tropical forest ecosystems.

AUTHOR CONTRIBUTIONS

SP and BB designed and conceived the study. SP produced the first draft of the manuscript, and both authors edited the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01045

REFERENCES

Aber, J., McDowell, W., Nadelhoffer, K., Magill, A., Berntson, G., Kamakea, M., et al. (1998). Nitrogen saturation in temperate forest ecosystems – hypotheses revisited. *Bioscience* 48, 921–934. doi: 10.2307/1313296

Alvarez-Clare, S., Mack, M. C., and Brooks, M. (2013). A direct test of nitrogen and phosphorus limitation to net primary productivity in a lowland tropical wet forest. *Ecology* 94, 1540–1551. doi: 10.1890/12-2128.1

Arai, H., Hadi, A., Darung, U., Limin, S. H., Takahashi, H., Hatano, R., et al. (2014). Land use change affects microbial biomass and fluxes of carbon dioxide and nitrous oxide in tropical peatlands. *Soil Sci. Plant Nutr.* 60, 423–434. doi: 10.1080/00380768.2014.903576

Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., et al. (2011). Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. *Glob. Change Biol.* 17, 1975–1989. doi: 10.1111/j.1365-2486.2010.02340.x

Avrahami, S., and Conrad, R. (2005). Cold-temperate climate: a factor for selection of ammonia oxidizers in upland soil? *Can. J. Microbiol.* 51, 709–714. doi: 10.1139/w05-045

Baggs, E. M. (2008). A review of stable isotope techniques for N_2O source partitioning in soils: recent progress, remaining challenges and future considerations. *Rapid Commun. Mass Spectrom.* 22, 1664–1672. doi: 10.1002/rcm.3456

Bai, E., Li, S., Xu, W., Li, W., Dai, W., and Jiang, P. (2013). A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. *New Phytol.* 199, 431–440. doi: 10.1111/nph.12252

Bardgett, R. D., Freeman, C., and Ostle, N. J. (2008). Microbial contributions to climate change through carbon cycle feedbacks. *ISME J.* 2, 805–814. doi: 10.1038/ismej.2008.58

Barron, A. R., Wurzburger, N., Bellenger, J. P., Wright, S. J., Kraepiel, A. M. L., and Hedin, L. O. (2008). Molybdenum limitation of asymbiotic nitrogen fixation in tropical forest soils. *Nat. Geosci.* 2, 42–45. doi: 10.1038/ngeo366

Bartossek, R., Nicol, G. W., Lanzen, A., Klenk, H. P., and Schleper, C. (2010). Homologues of nitrite reductases in ammonia-oxidizing archaea: diversity and genomic context. *Environ. Microbiol.* 12, 1075–1088. doi: 10.1111/j.1462-2920.2010.02153.x

Bedmar, E. J., Robles, E. F., and Delgado, M. J. (2005). The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochem. Soc. Trans.* 33, 141–144. doi: 10.1042/BST0330141

Benner, J. W., Conroy, S., Lunch, C. K., Toyoda, N., and Vitousek, P. M. (2007). Phosphorus fertilization increases the abundance and nitrogenase activity of the cyanolichen *Pseudocyphellaria crocata* in Hawaiian montane forests. *Biotropica* 39, 400–405. doi: 10.1111/j.1744-7429.2007.00267.x

Biggs, T. W., Dunne, T., and Martinelli, L. A. (2004). Natural controls and human impacts on stream nutrient concentrations in a deforested region of the Brazilian Amazon basin. *Biogeochemistry* 68, 227–257. doi: 10.1023/B:BIOG.0000025744.78309.2e

Bollmann, A., and Conrad, R. (1998). Influence of O_2 availability on NO and N_2O release by nitrification and denitrification in soils. *Glob. Change Biol.* 4, 387–396. doi: 10.1046/j.1365-2486.1998.00161.x

Bonan, G. B. (2008). Forests and climate change: forcings, feedbacks, and the climate benefits of forests. *Science* 320, 1444–1449. doi: 10.1126/science.1155121

Bossio, D. A., Girvan, M. S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., et al. (2005). Soil microbial community response to land use change in an agricultural landscape of western Kenya. *Microb. Ecol.* 49, 50–62. doi: 10.1007/s00248-003-0209-6

Bottomley, P. J., Taylor, A. E., and Myrold, D. D. (2012). A consideration of the relative contributions of different microbial subpopulations to the soil N cycle. *Front. Microbiol.* 3:373. doi: 10.3389/fmicb.2012.00373

Boyle-Yarwood, S. A., Bottomley, P. J., and Myrold, D. D. (2008). Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon. *Environ. Microbiol.* 10, 2956–2965. doi: 10.1111/j.1462-2920.2008.01600.x

Braker, G., and Conrad, R. (2011). Diversity, structure, and size of N_2O-producing microbial communities in soils-what matters for their functioning? *Adv. Appl. Microbiol.* 75, 33–70. doi: 10.1016/B978-0-12-387046-9.00002-5

Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., and Conrad, R. (2007). Impact of plant functional group, plant species, and sampling time on the composition of *nirK*-type denitrifier communities in soil. *Appl. Environ. Microbiol.* 73, 6876–6884. doi: 10.1128/AEM.01536-07

Brookshire, E. N. J., Gerber, S., Menge, D. N. L., and Hedin, L. O. (2012). Large losses of inorganic nitrogen from tropical rainforests suggest a lack of nitrogen limitation. *Ecol. Lett.* 15, 9–16. doi: 10.1111/j.1461-0248.2011.01701.x

Bruce, T., Martinez, I. B., Maia Neto, O., Vicente, A. C. P., Kruger, R. H., and Thompson, F. L. (2010). Bacterial community diversity in the Brazilian Atlantic forest soils. *Microb. Ecol.* 60, 840–849. doi: 10.1007/s00248-010-9750-2

Butterbach-Bahl, K., Kock, M., Willibald, G., Hewett, B., Buhagiar, S., Papen, H., et al. (2004). Temporal variations of fluxes of NO, NO_2, N_2O, CO_2, and CH_4 in a tropical rain forest ecosystem. *Glob. Biogeochem. Cycles* 18:GB3012. doi: 10.1029/2004gb002243

Cabello, P., Roldán, M. D., and Moreno-Vivián, C. (2004). Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* 150, 3527–3546. doi: 10.1099/mic.0.27303-0

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Carney, K. M., and Matson, P. A. (2006). The influence of tropical plant diversity and composition on soil microbial communities. *Microb. Ecol.* 52, 226–238. doi: 10.1007/s00248-006-9115-z

Carney, K. M., Matson, P. A., and Bohannan, B. J. M. (2004). Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. *Ecol. Lett.* 7, 684–694. doi: 10.1111/j.1461-0248.2004.00628.x

Cenciani, K., Lambais, M. R., Cerri, C. C., de Azevedo, L. C. B., and Feigl, B. J. (2009). Bacteria diversity and microbial biomass in forest, pasture and fallow soils in the southwestern Amazon basin. *Rev. Bras. Ciênc. Solo* 33, 907–916. doi: 10.1590/S0100-06832009000400015

Chen, R., Senbayram, M., Blagodatsky, S., Myachina, O., Dittert, K., Lin, X., et al. (2014). Soil C and N availability determine the priming effect: microbial N mining and stoichiometric decomposition theories. *Glob. Change Biol.* 20, 2356–2367. doi: 10.1111/gcb.12475

Chen, X. P., Zhu, Y. G., Xia, Y., Shen, J. P., and He, J. Z. (2008). Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? *Environ. Microbiol.* 10, 1978–1987. doi: 10.1111/j.1462-2920.2008.01613.x

Chen, X. Y., and Mulder, J. (2007). Atmospheric deposition of nitrogen at five subtropical forested sites in South China. *Sci. Total Environ.* 378, 317–330. doi: 10.1016/j.scitotenv.2007.02.028

Cleveland, C. C., and Townsend, A. R. (2006). Nutrient additions to a tropical rain forest drive substantial soil carbon dioxide losses to the atmosphere. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10316–10321. doi: 10.1073/pnas.0600989103

Cleveland, C. C., Townsend, A. R., Schimel, D. S., Fisher, H., Howarth, R. W., Hedin, L. O., et al. (1999). Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Glob. Biogeochem. Cycles* 13, 623–645. doi: 10.1029/1999GB900014

Cleveland, C. C., Townsend, A. R., Taylor, P., Alvarez-Clare, S., Bustamante, M. M. C., Chuyong, G., et al. (2011). Relationships among net primary productivity, nutrients and climate in tropical rain forest: a pan-tropical analysis. *Ecol. Lett.* 14, 939–947. doi: 10.1111/j.1461-0248.2011.01658.x

Colliver, B. B., and Stephenson, T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnol. Adv.* 18, 219–232. doi: 10.1016/S0734-9750(00)00035-5

Cong, J., Liu, X., Lu, H., Xu, H., Li, Y., Deng, Y., et al. (2015). Analyses of the influencing factors of soil microbial functional gene diversity in tropical rainforest based on GeoChip 5.0. *Genomics Data* 5, 397–398. doi: 10.1016/j.gdata.2015.07.010

Corre, M. D., Sueta, J. P., and Veldkamp, E. (2014). Nitrogen-oxide emissions from tropical forest soils exposed to elevated nitrogen input strongly interact with rainfall quantity and seasonality. *Biogeochemistry* 118, 103–120. doi: 10.1007/s10533-013-9908-3

Corre, M. D., Veldkamp, E., Arnold, J., and Wright, S. J. (2010). Impact of elevated N input on soil N cycling and losses in old-growth lowland and montane forests in Panama. *Ecology* 91, 1715–1729. doi: 10.1890/09-0274.1

Cusack, D. F., Silver, W. L., and McDowell, W. H. (2009). Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems* 12, 1299–1315. doi: 10.1007/s10021-009-9290-0

Cusack, D. F., Silver, W. L., Torn, M. S., Burton, S. D., and Firestone, M. K. (2011). Changes in microbial community characteristics and soil organic matter with nitrogen additions in two tropical forests. *Ecology* 92, 621–632. doi: 10.1890/10-0459.1

Cusack, D. F., Torn, M. S., McDowell, W. H., and Silver, W. L. (2010). The response of heterotrophic activity and carbon cycling to nitrogen additions and warming in two tropical soils. *Glob. Change Biol.* 16, 2555–2572. doi: 10.1111/j.1365-2486.2009.02131.x

da Jesus, E., Marsh, T. L., Tiedje, J. M., and de Moreira, F. M. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Dandie, C. E., Wertz, S., Leclair, C. L., Goyer, C., Burton, D. L., Patten, C. L., et al. (2011). Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. *FEMS Microbiol. Ecol.* 77, 69–82. doi: 10.1111/j.1574-6941.2011.01084.x

Davidson, E. A., Bustamante, M. M., and de Siqueira Pinto, A. (2001). Emissions of nitrous oxide and nitric oxide from soils of native and exotic ecosystems of the Amazon and Cerrado regions of Brazil. *ScientificWorldJournal* 1, 312–319. doi: 10.1100/tsw.2001.261

Davidson, E. A., Chorover, J., and Dail, D. B. (2003). A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis. *Glob. Change Biol.* 9, 228–236. doi: 10.1046/j.1365-2486.2003.00592.x

Davidson, E. A., de Carvalho, C. J. R., Figueira, A. M., Ishida, F. Y., Ometto, J. P. H. B., Nardoto, G. B., et al. (2007). Recuperation of nitrogen cycling in Amazonian forests following agricultural abandonment. *Nature* 447, 995–998. doi: 10.1038/nature05900

Davidson, E. A., Ishida, F. Y., and Nepstad, D. C. (2004). Effects of an experimental drought on soil emissions of carbon dioxide, methane, nitrous oxide, and nitric oxide in a moist tropical forest. *Glob. Change Biol.* 10, 718–730. doi: 10.1111/j.1365-2486.2004.00762.x

Davidson, E. A., Keller, M., Erickson, H. E., Verchot, L. V., and Veldkamp, E. (2000). Testing a conceptual model of soil emissions of nitrous and nitric oxides. *Bioscience* 50, 667–680. doi: 10.1641/0006-3568(2000)050[0667:TACMOS]2.0.CO;2

de Boer, W., and Kowalchuk, G. A. (2001). Nitrification in acid soils: microorganisms and mechanisms. *Soil Biol. Biochem.* 33, 853–866. doi: 10.1016/S0038-0717(00)00247-9

de Gannes, V., Eudoxie, G., and Hickey, W. J. (2014). Impacts of edaphic factors on communities of ammonia-oxidizing archaea, ammonia-oxidizing bacteria and nitrification in tropical soils. *PLoS ONE* 9:e89568. doi: 10.1371/journal.pone.0089568

DeAngelis, K. M., Silver, W. L., Thompson, A. W., and Firestone, M. K. (2010). Microbial communities acclimate to recurring changes in soil redox potential status. *Environ. Microbiol.* 12, 3137–3149. doi: 10.1111/j.1462-2920.2010.02286.x

Demanèche, S., Philippot, L., David, M. M., Navarro, E., Vogel, T. M., and Simonet, P. (2009). Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. *Appl. Environ. Microbiol.* 75, 534–537. doi: 10.1128/AEM.01706-08

Diffenbaugh, N. S., and Scherer, M. (2011). Observational and model evidence of global emergence of permanent, unprecedented heat in the 20th and 21st centuries. *Clim. Change* 107, 615–624. doi: 10.1007/s10584-011-0112-y

Erguder, T. H., Boon, N., Wittebolle, L., Marzorati, M., and Verstraete, W. (2009). Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiol. Rev.* 33, 855–869. doi: 10.1111/j.1574-6976.2009.00179.x

Erisman, J. W., Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M. R., et al. (2013). Consequences of human modification of the global nitrogen cycle. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368:20130116. doi: 10.1098/rstb.2013.0116

Fabian, P., Kohlpaintner, M., and Rollenbeck, R. (2005). Biomass burning in the Amazon-fertilizer for the mountaineous rain forest in Ecuador. *Environ. Sci. Pollut. Res. Int.* 12, 290–296. doi: 10.1065/espr2005.07.272

Fang, Y., Yoh, M., Koba, K., Zhu, W., Takebayashi, Y., Xiao, Y., et al. (2011). Nitrogen deposition and forest nitrogen cycling along an urban-rural transect in southern China. *Glob. Change Biol.* 17, 872–885. doi: 10.1111/j.1365-2486.2010.02283.x

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology* 88, 1354–1364. doi: 10.1890/05-1839

Fierer, N., Carney, K. M., Horner-Devine, M. C., and Megonigal, J. P. (2009a). The biogeography of ammonia-oxidizing bacterial communities in soil. *Microb. Ecol.* 58, 435–445. doi: 10.1007/s00248-009-9517-9

Fierer, N., Grandy, A. S., Six, J., and Paul, E. A. (2009b). Searching for unifying principles in soil ecology. *Soil Biol. Biochem.* 41, 2249–2256. doi: 10.1016/j.soilbio.2009.06.009

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 103, 626–631. doi: 10.1073/pnas.0507535103

Fierer, N., Lauber, C. L., Ramirez, K. S., Zaneveld, J., Bradford, M. A., and Knight, R. (2012a). Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *ISME J.* 6, 1007–1017. doi: 10.1038/ismej.2011.159

Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., et al. (2012b). Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 21390–21395. doi: 10.1073/pnas.1215210110

Firestone, M. K., and Davidson, E. (1989). "Microbiological basis of NO and N_2O production and consumption in soil," in *Exchange of Trace Gases Between Terrestrial Ecosystems and the Atmosphere*, eds M. O. Andreae and D. S. Schimel (Chichester: John Wiley & Sons), 7–21.

Foley, J., Costa, M., Delire, C., Ramankutty, N., and Snyder, P. (2003). Green Surprise? How terrestrial ecosystems could effect earths climate. *Front. Ecol. Environ.* 1:38–44. doi: 10.1890/1540-9295(2003)001[0038:GSHTEC]2.0.CO;2

Gaby, J. C., and Buckley, D. H. (2011). A global census of nitrogenase diversity. *Environ. Microbiol.* 13, 1790–1799. doi: 10.1111/j.1462-2920.2011.02488.x

Gaby, J. C., and Buckley, D. H. (2012). A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLoS ONE* 7:e42149. doi: 10.1371/journal.pone.0042149

Galford, G. L., Melillo, J. M., Kicklighter, D. W., Cronin, T. W., Cerri, C. E. P., Mustard, J. F., et al. (2010). Greenhouse gas emissions from alternative futures of deforestation and agricultural management in the southern Amazon. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19649–19654. doi: 10.1073/pnas.1000780107

Galloway, J. N., Aber, J. D., Erisman, J. W., Seitzinger, S. P., Howarth, W., Cowling, E. B., et al. (2003). The nitrogen cascade. *Bioscience* 53, 341–356. doi: 10.1641/0006-3568(2003)053[0341:TNC]2.0.CO;2

Galloway, J. N., Dentener, F., and Capone, D. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry* 70, 153–226. doi: 10.1007/s10533-004-0370-0

Gao, W., Yang, H., Kou, L., and Li, S. (2015). Effects of nitrogen deposition and fertilization on N transformations in forest soils: a review. *J. Soils Sediments* 15, 863–879. doi: 10.1007/s11368-015-1064-z

Gharahi Ghehi, N., Werner, C., Cizungu Ntaboba, L., Mbonigaba Muhinda, J. J., Van Ranst, E., Butterbach-Bahl, K., et al. (2012). Spatial variations of nitrogen trace gas emissions from tropical mountain forests in Nyungwe, Rwanda. *Biogeosciences* 9, 1451–1463. doi: 10.5194/bg-9-1451-2012

Gibbs, H. K., Ruesch, A. S., Achard, F., Clayton, M. K., Holmgren, P., Ramankutty, N., et al. (2010). Tropical forests were the primary sources of new agricultural land in the 1980s and 1990s. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16732–16737. doi: 10.1073/pnas.0910275107

Gruber, N., and Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature* 451, 293–296. doi: 10.1038/nature06592

Gubry-Rangin, C., Nicol, G. W., and Prosser, J. I. (2010). Archaea rather than bacteria control nitrification in two agricultural acidic soils. *FEMS Microbiol. Ecol.* 74, 566–574. doi: 10.1111/j.1574-6941.2010.00971.x

Hall, S. J., and Matson, P. A. (1999). Nitrogen oxide emissions after nitrogen additions in tropical forests. *Nature* 400, 152–155. doi: 10.1038/22094

Hall, S. J., and Matson, P. A. (2003). Nutrient status of tropical rain forests influences soil N dynamics after N additions. *Ecol. Monogr.* 73, 107–129. doi: 10.1890/0012-9615(2003)073[0107:NSOTRF]2.0.CO;2

Hallin, S., Jones, C. M., Schloter, M., and Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J.* 3, 597–605. doi: 10.1038/ismej.2008.128

Harrington, R. A., Fownes, J. H., and Vitousek, P. M. (2001). Production and resource use efficiencies in N- and P-limited tropical forests: a comparison of responses to long-term fertilization. *Ecosystems* 4, 646–657. doi: 10.1007/s10021-001-0034-z

Hawksworth, D. L. (2012). Global species numbers of fungi: are tropical studies and molecular approaches contributing to a more robust estimate? *Biodivers. Conserv.* 21, 2425–2433. doi: 10.1007/s10531-012-0335-x

Hayatsu, M., Tago, K., and Saito, M. (2008). Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Sci. Plant Nutr.* 54, 33–45. doi: 10.1111/j.1747-0765.2007.00195.x

He, J.-Z., Shen, J.-P., Zhang, L.-M., Zhu, Y.-G., Zheng, Y.-M., Xu, M.-G., et al. (2007). Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environ. Microbiol.* 9, 2364–2374. doi: 10.1111/j.1462-2920.2007.01358.x

Hedin, L. O., Brookshire, E. N. J., Menge, D. N. L., and Barron, A. R. (2009). The nitrogen paradox in tropical forest ecosystems. *Annu. Rev. Ecol. Evol. Syst.* 40, 613–635. doi: 10.1146/annurev.ecolsys.37.091305.110246

Hedin, L. O., Vitousek, P. M., and Matson, P. A. (2003). Nutrient losses over four million years of tropical forest development. *Ecology* 84, 2231–2255. doi: 10.1890/02-4066

Henry, S., Baudoin, E., López-Gutiérrez, J. C., Martin-Laurent, F., Brauman, A., and Philippot, L. (2004). Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J. Microbiol. Methods* 59, 327–335. doi: 10.1016/j.mimet.2004.07.002

Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006). Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl. Environ. Microbiol.* 72, 5181–5189. doi: 10.1128/AEM.00231-06

Hicks, W. T., Harmon, M. E., and Griffiths, R. P. (2003). Abiotic controls on nitrogen fixation and respiration in selected woody debris from the Pacific Northwest, USA. *Ecoscience* 10, 66–73.

Hietz, P., Turner, B. L., Wanek, W., Richter, A., Nock, C. A., and Wright, S. J. (2011). Long-term change in the nitrogen cycle of tropical forests. *Science* 334, 664–666. doi: 10.1126/science.1211979

Hofstra, N., and Bouwman, A. F. (2005). Denitrification in agricultural soils: summarizing published data and estimating global annual rates. *Nutr. Cycl. Agroecosyst.* 72, 267–278. doi: 10.1007/s10705-005-3109-y

Homeier, J., Hertel, D., Camenzind, T., Cumbicus, N. L., Maraun, M., Martinson, G. O., et al. (2012). Tropical andean forests are highly susceptible to nutrient inputs-rapid effects of experimental N and P addition to an Ecuadorian montane forest. *PLoS ONE* 7:e47128. doi: 10.1371/journal.pone.0047128

Houlton, B. Z., and Bai, E. (2009). Imprint of denitrifying bacteria on the global terrestrial biosphere. *Proc. Natl. Acad. Sci. U.S.A.* 106, 21713–21716. doi: 10.1073/pnas.0912111106

Houlton, B. Z., Sigman, D. M., and Hedin, L. O. (2006). Isotopic evidence for large gaseous nitrogen losses from tropical rainforests. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8745–8750. doi: 10.1073/pnas.0510185103

Houlton, B. Z., Wang, Y.-P., Vitousek, P. M., and Field, C. B. (2008). A unifying framework for dinitrogen fixation in the terrestrial biosphere. *Nature* 454, 327–330. doi: 10.1038/nature07028

Humbert, S., Tarnawski, S., Fromin, N., Mallet, M.-P., Aragno, M., and Zopfi, J. (2010). Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. *ISME J.* 4, 450–454. doi: 10.1038/ismej.2009.125

IPCC (2013). *Climate Change 2013: The Physical Science Basis*. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press.

Isobe, K., Koba, K., Suwa, Y., Ikutani, J., Fang, Y., Yoh, M., et al. (2012). High abundance of ammonia-oxidizing archaea in acidified subtropical forest soils in southern China after long-term N deposition. *FEMS Microbiol. Ecol.* 80, 193–203. doi: 10.1111/j.1574-6941.2011.01294.x

Jia, Z., and Conrad, R. (2009). Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. *Environ. Microbiol.* 11, 1658–1671. doi: 10.1111/j.1462-2920.2009.01891.x

Jiang, X., Xin, X., Li, S., Zhou, J., Zhu, T., Müller, C., et al. (2015). Effects of Fe oxide on N transformations in subtropical acid soils. *Sci. Rep.* 5:8615. doi: 10.1038/srep08615

Jones, C. M., Graf, D. R. H., Bru, D., Philippot, L., and Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J.* 7, 417–426. doi: 10.1038/ismej.2012.125

Jones, C. M., Stres, B., Rosenquist, M., and Hallin, S. (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Mol. Biol. Evol.* 25, 1955–1966. doi: 10.1093/molbev/msn146

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., and Philippot, L. (2006). Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* 72, 5957–5962. doi: 10.1128/AEM.00439-06

Kaspari, M., Garcia, M. N., Harms, K. E., Santana, M., Wright, S. J., and Yavitt, J. B. (2008). Multiple nutrients limit litterfall and decomposition in a tropical forest. *Ecol. Lett.* 11, 35–43. doi: 10.1111/j.1461-0248.2007.01124.x

Katsuyama, C., Kondo, N., Suwa, Y., Yamagishi, T., Itoh, M., Ohte, N., et al. (2008). Denitrification activity and relevant bacteria revealed by nitrite reductase gene fragments in soil of temperate mixed forest. *Microbes Environ.* 23, 337–345. doi: 10.1264/jsme2.ME08541

Kim, J. S., Sparovek, G., Longo, R. M., De Melo, W. J., and Crowley, D. (2007). Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. *Soil Biol. Biochem.* 39, 684–690. doi: 10.1016/j.soilbio.2006.08.010

Klemedtsson, L., Von Arnold, K., Weslien, P., and Gundersen, P. (2005). Soil CN ratio as a scalar parameter to predict nitrous oxide emissions. *Glob. Change Biol.* 11, 1142–1147. doi: 10.1111/j.1365-2486.2005.00973.x

Koch, O., Tscherko, D., and Kandeler, E. (2007). Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. *Glob. Biogeochem. Cycles* 21:GB4017. doi: 10.1029/2007GB002983

Kool, D. M., Wrage, N., Zechmeister-Boltenstern, S., Pfeffer, M., Brus, D., Oenema, O., et al. (2010). Nitrifier denitrification can be a source of N_2O from soil: a revised approach to the dual-isotope labelling method. *Eur. J. Soil Sci.* 61, 759–772. doi: 10.1111/j.1365-2389.2010.01270.x

Kowalchuk, G. A., and Stephen, J. R. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu. Rev. Microbiol.* 55, 485–529. doi: 10.1146/annurev.micro.55.1.485

Krashevska, V., Klarner, B., Widyastuti, R., Maraun, M., and Scheu, S. (2015). Impact of tropical lowland rainforest conversion into rubber and oil palm plantations on soil microbial communities. *Biol. Fertil. Soils* 51, 697–705. doi: 10.1007/s00374-015-1021-4

Krashevska, V., Sandmann, D., Maraun, M., and Scheu, S. (2012). Consequences of exclusion of precipitation on microorganisms and microbial consumers in montane tropical rainforests. *Oecologia* 170, 1067–1076. doi: 10.1007/s00442-012-2360-6

Krashevska, V., Sandmann, D., Maraun, M., and Scheu, S. (2014). Moderate changes in nutrient input alter tropical microbial and protist communities and belowground linkages. *ISME J.* 8, 1126–1134. doi: 10.1038/ismej.2013.209

Lammel, D. R., Feigl, B. J., Cerri, C. C., and Nüsslein, K. (2015). Specific microbial gene abundances and soil parameters contribute to C, N, and greenhouse gas process rates after land use change in Southern Amazonian Soils. *Front. Microbiol.* 6:1057. doi: 10.3389/fmicb.2015.01057

Lee-Cruz, L., Edwards, D. P., Tripathi, B. M., and Adams, J. M. (2013). Impact of logging and forest conversion to oil palm plantations on soil bacterial communities in Borneo. *Appl. Environ. Microbiol.* 79, 7290–7297. doi: 10.1128/AEM.02541-13

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., et al. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442, 806–809. doi: 10.1038/nature04983

Levičnik-Höfferle, Š, Nicol, G. W., Ausec, L., Mandić-Mulec, I., and Prosser, J. I (2012). Stimulation of thaumarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen. *FEMS Microbiol. Ecol.* 80, 114–123. doi: 10.1111/j.1574-6941.2011.01275.x

Levy-Booth, D. J., Prescott, C. E., and Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biol. Biochem.* 75, 11–25. doi: 10.1016/j.soilbio.2014.03.021

Liu, B., Mørkved, P. T., Frostegård, Å, and Bakken, L. R. (2010). Denitrification gene pools, transcription and kinetics of NO, N_2O and N_2 production as affected by soil pH. *FEMS Microbiol. Ecol.* 72, 407–417. doi: 10.1111/j.1574-6941.2010.00856.x

Liu, L., and Greaver, T. L. (2009). A review of nitrogen enrichment effects on three biogenic GHGs: the CO_2 sink may be largely offset by stimulated N_2O and CH_4 emission. *Ecol. Lett.* 12, 1103–1117. doi: 10.1111/j.1461-0248.2009.01351.x

Liu, L., Zhang, T., Gilliam, F. S., Gundersen, P., Zhang, W., Chen, H., et al. (2013a). Interactive effects of nitrogen and phosphorus on soil microbial communities in a tropical forest. *PLoS ONE* 8:e61188. doi: 10.1371/journal.pone.0061188

Liu, X., Chen, C. R., Wang, W. J., Hughes, J. M., Lewis, T., Hou, E. Q., et al. (2013b). Soil environmental factors rather than denitrification gene abundance control N_2O fluxes in a wet sclerophyll forest with different burning frequency. *Soil Biol. Biochem.* 57, 292–300. doi: 10.1016/j.soilbio.2012.10.009

Lohse, K. A., and Matson, P. (2005). Consequences of nitrogen additions for soil losses from wet tropical forests. *Ecol. Appl.* 15, 1629–1648. doi: 10.1890/03-5421

Lu, X., Mao, Q., Gilliam, F. S., Luo, Y., and Mo, J. (2014). Nitrogen deposition contributes to soil acidification in tropical ecosystems. *Glob. Change Biol.* 20, 3790–3801. doi: 10.1111/gcb.12665

Lu, X.-K., Mo, J.-M., Gundersern, P., Zhu, W.-X., Zhou, G.-Y., Li, D.-J., et al. (2009). Effect of simulated N deposition on soil exchangeable cations in three forest types of subtropical China. *Pedosphere* 19, 189–198. doi: 10.1016/S1002-0160(09)60108-9

Macrae, A., Coelho, R. R. R., Peixoto, R., and Rosado, A. S. (2013). "Tropical soil microbial communities," in *The Prokaryotes: Prokaryotic Communities and Ecophysiology*, eds E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson (Heidelberg: Springer Berlin), 85–95. doi: 10.1007/978-3-642-30123-0_115

Martinelli, L. A., Piccolo, M. C., Townsend, A. R., Vitousek, P. M., Cuevas, E., MacDowell, W., et al. (1999). Nitrogen stable isotopic composition of leaves and soil: tropical versus temperate forests. *Biogeochemistry* 46, 45–65. doi: 10.1023/A:1006100128782

Martinson, G. O., Corre, M. D., and Veldkamp, E. (2012). Responses of nitrous oxide fluxes and soil nitrogen cycling to nutrient additions in montane forests along an elevation gradient in southern Ecuador. *Biogeochemistry* 112, 625–636. doi: 10.1007/s10533-012-9753-9

Matson, P. A., McDowell, W. H., Townsend, A. R., and Vitousek, P. M. (1999). The globalization of N deposition: ecosystem consequences in tropical environments. *Biogeochemistry* 46, 67–83. doi: 10.1007/BF01007574

Matzek, V., and Vitousek, P. (2003). Nitrogen fixation in bryophytes, lichens, and decaying wood along a soil-age gradient in Hawaiian montane rain forest. *Biotropica* 35, 12–19. doi: 10.1646/0006-3606(2003)035[0012:NFIBLA]2.0.CO;2

McGrath, D. A., Smith, C. K., Gholz, H. L., and Oliveira, F. D. (2001). Effects of land-use change on soil nutrient dynamics in Amazonia. *Ecosystems* 4, 625–645. doi: 10.1007/s10021-001-0033-0

McGuire, K. L., Fierer, N., Bateman, C., Treseder, K. K., and Turner, B. L. (2012). Fungal community composition in neotropical rain forests: the influence of tree diversity and precipitation. *Microb. Ecol.* 63, 804–812. doi: 10.1007/s00248-011-9973-x

Mirza, B. S., Potisap, C., Nüsslein, K., Bohannan, B. J. M., and Rodriguesa, J. L. M. (2014). Response of free-living nitrogen-fixing microorganisms to land use change in the amazon rainforest. *Appl. Environ. Microbiol.* 80, 281–288. doi: 10.1128/AEM.02362-13

Mooshammer, M., Wanek, W., Schnecker, J., Wild, B., Leitner, S., Hofhansl, F., et al. (2012). Stoichiometric controls of nitrogen and phosphorus cycling in decomposing beech leaf litter. *Ecology* 93, 770–782. doi: 10.1890/11-0721.1

Morley, N., Baggs, E. M., Dörsch, P., and Bakken, L. (2008). Production of NO, N_2O and N_2 by extracted soil bacteria, regulation by NO_2- and O_2 concentrations. *FEMS Microbiol. Ecol.* 65, 102–112. doi: 10.1111/j.1574-6941.2008.00495.x

Moseman, S. M., Zhang, R., Qian, P. Y., and Levin, L. A. (2009). Diversity and functional responses of nitrogen-fixing microbes to three wetland invasions. *Biol. Invasions* 11, 225–239. doi: 10.1007/s10530-008-9227-0

Mueller, R. C., Paula, F. S., Mirza, B. S., Rodrigues, J. L., Nüsslein, K., and Bohannan, B. J. (2014). Links between plant and fungal communities across a deforestation chronosequence in the Amazon rainforest. *ISME J.* 8, 1548–1550. doi: 10.1038/ismej.2013.253

Mulder, A., van de Graaf, A. A., and Robertson, L. A. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol. Ecol.* 16, 177–183. doi: 10.1016/0168-6496(94)00081-7

Murty, D., Kirschbaum, M. U. F., McMurtrie, R. E., and McGilvray, H. (2002). Does conversion of forest to agricultural land change soil carbon and nitrogen? A review of the literature. *Glob. Change Biol.* 8, 105–123. doi: 10.1046/j.1354-1013.2001.00459.x

Neill, C., Steudler, P. A., Garcia-Montiel, D. C., Melillo, J. M., Feigl, B. J., Piccolo, M. C., et al. (2005). Rates and controls of nitrous oxide and nitric oxide emissions following conversion of forest to pasture in Rondônia. *Nutr. Cycl. Agroecosyst.* 71, 1–15. doi: 10.1007/s10705-004-0378-9

Nicol, G. W., Leininger, S., Schleper, C., and Prosser, J. I. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia

oxidizing archaea and bacteria. *Environ. Microbiol.* 10, 2966–2978. doi: 10.1111/j.1462-2920.2008.01701.x

Nicol, G. W., and Schleper, C. (2006). Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol.* 14, 207–212. doi: 10.1016/j.tim.2006.03.004

Palmer, K., Biasi, C., and Horn, M. A. (2012). Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic peat soils in arctic tundra. *ISME J.* 6, 1058–1077. doi: 10.1038/ismej.2011.172

Paterson, E. (2003). Importance of rhizodeposition in the coupling of plant and microbial productivity. *Eur. J. Soil Sci.* 54, 741–750. doi: 10.1046/j.1351-0754.2003.0557.x

Paula, F. S., Rodrigues, J. L. M., Zhou, J., Wu, L., Mueller, R. C., Mirza, B. S., et al. (2014). Land use change alters functional gene diversity, composition and abundance in Amazon forest soil microbial communities. *Mol. Ecol.* 23, 2988–2999. doi: 10.1111/mec.12786

Pearson, H. L., and Vitousek, P. M. (2002). Soil phosphorus fractions and symbiotic nitrogen fixation across a substrate-age gradient in Hawaii. *Ecosystems* 5, 587–596. doi: 10.1007/s10021-002-0172-y

Pereira e Silva, M. C., Schloter-Hai, B., Schloter, M., van Elsas, J. D., and Salles, J. F. (2013). Temporal dynamics of abundance and composition of nitrogen-fixing communities across agricultural soils. *PLoS ONE* 8:e74500. doi: 10.1371/journal.pone.0074500

Pester, M., Rattei, T., Flechl, S., Gröngröft, A., Richter, A., Overmann, J., et al. (2012). *amoA*-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of *amoA* genes from soils of four different geographic regions. *Environ. Microbiol.* 14, 525–539. doi: 10.1111/j.1462-2920.2011.02666.x

Pett-Ridge, J., Petersen, D. G., Nuccio, E., and Firestone, M. K. (2013). Influence of oxic/anoxic fluctuations on ammonia oxidizers and nitrification potential in a wet tropical soil. *FEMS Microbiol. Ecol.* 85, 179–194. doi: 10.1111/1574-6941.12111

Pett-Ridge, J., Silver, W. L., and Firestone, M. K. (2006). Redox fluctuations frame microbial community impacts on N-cycling rates in a humid tropical forest soil. *Biogeochemistry* 81, 95–110. doi: 10.1007/s10533-006-9032-8

Philippot, L., Andersson, S. G. E., Battin, T. J., Prosser, J. I., Schimel, J. P., Whitman, W. B., et al. (2010). The ecological coherence of high bacterial taxonomic ranks. *Nat. Rev. Microbiol.* 8, 523–529. doi: 10.1038/nrmicro2367

Philippot, L., Andert, J., Jones, C. M., Bru, D., and Hallin, S. (2011). Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Glob. Chang. Biol.* 17, 1497–1504. doi: 10.1111/j.1365-2486.2010.02334.x

Philippot, L., Hallin, S., and Schloter, M. (2007). Ecology of denitrifying prokaryotes in agricultural soil. *Adv. Agron.* 96, 249–305. doi: 10.1016/S0065-2113(07)96003-4

Philippot, L., Spor, A., Hénault, C., Bru, D., Bizouard, F., Jones, C. M., et al. (2013). Loss in microbial diversity affects nitrogen cycling in soil. *ISME J.* 7, 1609–1619. doi: 10.1038/ismej.2013.34

Prosser, J. I., Bohannan, B. J. M., Curtis, T. P., Ellis, R. J., Firestone, M. K., Freckleton, R. P., et al. (2007). The role of ecological theory in microbial ecology. *Nat. Rev. Microbiol.* 5, 384–392. doi: 10.1038/nrmicro1643

Ramirez, K. S., Craine, J. M., and Fierer, N. (2012). Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Glob. Change Biol.* 18, 1918–1927. doi: 10.1111/j.1365-2486.2012.02639.x

Ranjan, K., Paula, F. S., Mueller, R. C., Jesus, E. D. C., Cenciani, K., Bohannan, B. J. M., et al. (2015). Forest-to-pasture conversion increases the diversity of the phylum Verrucomicrobia in Amazon rainforest soils. *Front. Microbiol.* 6:779. doi: 10.3389/fmicb.2015.00779

Reay, D. S., Dentener, F., Smith, P., Grace, J., and Feely, R. A. (2008). Global nitrogen deposition and carbon sinks. *Nat. Geosci.* 1, 430–437. doi: 10.1038/ngeo230

Reed, S. C., Cleveland, C. C., and Townsend, A. R. (2007). Controls over leaf litter and soil nitrogen fixation in two lowland tropical rain forests. *Biotropica* 39, 585–592. doi: 10.1111/j.1744-7429.2007.00310.x

Reed, S. C., Cleveland, C. C., and Townsend, A. R. (2013). Relationships among phosphorus, molybdenum and free-living nitrogen fixation in tropical rain forests: results from observational and experimental analyses. *Biogeochemistry* 114, 135–147. doi: 10.1007/s10533-013-9835-3

Reed, S. C., Townsend, A. R., Cleveland, C. C., and Nemergut, D. R. (2010). Microbial community shifts influence patterns in tropical forest nitrogen fixation. *Oecologia* 164, 521–531. doi: 10.1007/s00442-010-1649-6

Reverchon, F., Bai, S. H., Liu, X., and Blumfield, T. J. (2015). Tree plantation systems influence nitrogen retention and the abundance of nitrogen functional genes in the solomon islands. *Front. Microbiol.* 6:1439. doi: 10.3389/fmicb.2015.01439

Richardson, D., Felgate, H., Watmough, N., Thomson, A., and Baggs, E. (2009). Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle – could enzymic regulation hold the key? *Trends Biotechnol.* 27, 388–397. doi: 10.1016/j.tibtech.2009.03.009

Robertson, G. P., and Tiedje, J. M. (1988). Deforestation alters denitrification in a lowland tropical rain forest. *Nature* 336, 756–759. doi: 10.1038/336756a0

Rodrigues, J. L. M., Pellizari, V. H., Mueller, R., Baek, K., Jesus, E. D. C., Paula, F. S., et al. (2013). Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 110, 988–993. doi: 10.1073/pnas.1220608110

Roesch, L. F. W., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K. M., Kent, A. D., et al. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J.* 1, 283–290. doi: 10.1038/ismej.2007.53

Rosch, C., Mergel, A., and Bothe, H. (2002). Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.* 68, 3818–3829. doi: 10.1128/AEM.68.8.3818

Rotthauwe, J. H., Witzel, K. P., and Liesack, W. (1997). The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712.

Rousk, J., and Bååth, E. (2007). Fungal and bacterial growth in soil with plant materials of different C/N ratios. *FEMS Microbiol. Ecol.* 62, 258–267. doi: 10.1111/j.1574-6941.2007.00398.x

Sala, O. E., Chapin, F. S., Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., et al. (2000). Global biodiversity scenarios for the year 2100. *Science* 287, 1770–1774. doi: 10.1126/science.287.5459.1770

Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-García, C., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19709–19714. doi: 10.1073/pnas.1211238109

Schimel, J. P., and Schaeffer, S. M. (2012). Microbial control over carbon cycling in soil. *Front. Microbiol.* 3:348. doi: 10.3389/fmicb.2012.00348

Sey, B. K., Manceur, A. M., Whalen, J. K., Gregorich, E. G., and Rochette, P. (2008). Small-scale heterogeneity in carbon dioxide, nitrous oxide and methane production from aggregates of a cultivated sandy-loam soil. *Soil Biol. Biochem.* 40, 2468–2473. doi: 10.1016/j.soilbio.2008.05.012

Shaw, L. J., Nicol, G. W., Smith, Z., Fear, J., Prosser, J. I., and Baggs, E. M. (2006). *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ. Microbiol.* 8, 214–222. doi: 10.1111/j.1462-2920.2005.00882.x

Shen, J. P., Zhang, L. M., Zhu, Y. G., Zhang, J. B., and He, J. Z. (2008). Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environ. Microbiol.* 10, 1601–1611. doi: 10.1111/j.1462-2920.2008.01578.x

Shoun, H., Kim, D. H., Uchiyama, H., and Sugiyama, J. (1992). Denitrification by fungi. *FEMS Microbiol. Lett.* 94, 277–282. doi: 10.1016/0378-1097(92)90643-3

Shrestha, N. K., Hadano, S., Kamachi, T., and Okura, I. (2002). Dinitrogen production from ammonia by *Nitrosomonas europaea*. *Appl. Catal. A Gen.* 237, 33–39. doi: 10.1016/S0926-860X(02)00279-X

Silver, W. L., Herman, D. J., and Firestone, M. K. (2001). Dissimilatory nitrate reduction to ammonium in upland tropical forest soils. *Ecology* 82, 2410–2416. doi: 10.1890/0012-9658(2001)082[2410:DNRTAI]2.0.CO;2

Šimek, M., Jíšová, L., and Hopkins, D. W. (2002). What is the so-called optimum pH for denitrification in soil? *Soil Biol. Biochem.* 34, 1227–1234. doi: 10.1016/S0038-0717(02)00059-7

Singh, B. K., Bardgett, R. D., Smith, P., and Reay, D. S. (2010). Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nat. Rev. Microbiol.* 8, 779–790. doi: 10.1038/nrmicro2439

Smith, C. J., Nedwell, D. B., Dong, L. F., and Osborn, A. M. (2007). Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Appl. Environ. Microbiol.* 73, 3612–3622. doi: 10.1128/AEM.02894-06

Sonthiphand, P., Hall, M. W., and Neufeld, J. D. (2014). Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria. *Front. Microbiol.* 5:399. doi: 10.3389/fmicb.2014.00399

Spott, O., Russow, R., and Stange, C. F. (2011). Formation of hybrid N_2O and hybrid N_2 due to codenitrification: first review of a barely considered process of microbially mediated N-nitrosation. *Soil Biol. Biochem.* 43, 1995–2011. doi: 10.1016/j.soilbio.2011.06.014

Stehfest, E., and Bouwman, L. (2006). N_2O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions. *Nutr. Cycl. Agroecosyst.* 74, 207–228. doi: 10.1007/s10705-006-9000-7

Stone, M. M., Kan, J., and Plante, A. F. (2015). Parent material and vegetation influence bacterial community structure and nitrogen functional genes along deep tropical soil profiles at the Luquillo Critical Zone Observatory. *Soil Biol. Biochem.* 80, 273–282. doi: 10.1016/j.soilbio.2014.10.019

Stopnišek, N., Gubry-Rangin, C., Höfferle, Š, Nicol, G. W., Mandič-Mulec, I., and Prosser, J. I (2010). *Thaumarchaeal* ammonia oxidation in an acidic forest peat soil is not influenced by ammonium amendment. *Appl. Environ. Microbiol.* 76, 7626–7634. doi: 10.1128/AEM.00595-10

Stork, N. E., Coddington, J. A., Colwell, R. K., Chazdon, R. L., Dick, C. W., Peres, C. A., et al. (2009). Vulnerability and resilience of tropical forest species to land-use change. *Conserv. Biol.* 23, 1438–1447. doi: 10.1111/j.1523-1739.2009.01335.x

Taketani, R. G., and Tsai, S. M. (2010). The influence of different land uses on the structure of archaeal communities in Amazonian anthrosols based on 16S rRNA and *amoA* genes. *Microb. Ecol.* 59, 734–743. doi: 10.1007/s00248-010-9638-1

Tanner, E. V. J., Vitousek, P. M., and Cuevas, E. (1998). Experimental investigation of nutrient limitation of forest growth on wet tropical mountains. *Ecology* 79, 10–22. doi: 10.1890/0012-9658(1998)079[0010:EIONLO]2.0.CO;2

Taylor, A. E., Zeglin, L. H., Wanzek, T. A., Myrold, D. D., and Bottomley, P. J. (2012). Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. *ISME J.* 6, 2024–2032. doi: 10.1038/ismej.2012.51

Templer, P. H., Silver, W. L., Pett-Ridge, J., DeAngelis, K. M., and Firestone, M. K. (2008). Plant and microbial controls on nitrogen retention and loss in a humid tropical forest. *Ecology* 89, 3030–3040. doi: 10.1890/07-1631.1

Thornton, P. E., Doney, S. C., Lindsay, K., Moore, J. K., Mahowald, N., Randerson, J. T., et al. (2009). Carbon-nitrogen interactions regulate climate-carbon cycle feedbacks: results from an atmosphere-ocean general circulation model. *Biogeosciences* 6, 2099–2120. doi: 10.5194/bg-6-2099-2009

Tiedje, J. M. (1988). "Ecology of denitrification and dissimilatory nitrate reduction to ammonium," in *Environmental Microbiology of Anaerobes*, ed. A. J. B. Zehnder (New York, NY: John Wiley and Sons), 179–244.

Tiedje, J. M., Sexstone, A. J., Myrold, D. D., and Robinson, J. A. (1983). Denitrification: ecological niches, competition and survival. *Antonie Van Leeuwenhoek* 48, 569–583. doi: 10.1007/BF00399542

Townsend, A. R., Asner, G. P., and Cleveland, C. C. (2008). The biogeochemical heterogeneity of tropical forests. *Trends Ecol. Evol.* 23, 424–431. doi: 10.1016/j.tree.2008.04.009

Townsend, A. R., Cleveland, C. C., Houlton, B. Z., Alden, C. B., and White, J. W. C. (2011). Multi-element regulation of the tropical forest carbon cycle. *Front. Ecol. Environ.* 9:9–17. doi: 10.1890/100047

Treseder, K. K., Balser, T. C., Bradford, M. A., Brodie, E. L., Dubinsky, E. A., Eviner, V. T., et al. (2011). Integrating microbial ecology into ecosystem models: challenges and priorities. *Biogeochemistry* 109, 7–18. doi: 10.1007/s10533-011-9636-5

Treusch, A. H., Leininger, S., Kletzin, A., Schuster, S. C., Klenk, H.-P., and Schleper, C. (2005). Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* 7, 1985–1995. doi: 10.1111/j.1462-2920.2005.00906.x

Tu, L., Chen, G., Peng, Y., Hu, H., Hu, T., Zhang, J., et al. (2014). Soil biochemical responses to nitrogen addition in a bamboo forest. *PLoS ONE* 9:e102315. doi: 10.1371/journal.pone.0102315

Tu, L., Hu, T., Zhang, J., Huang, L., Xiao, Y., Chen, G., et al. (2013). Nitrogen distribution and cycling through water flows in a subtropical bamboo forest under high level of atmospheric deposition. *PLoS ONE* 8:e75862. doi: 10.1371/journal.pone.0075862

Tu, Q., Zhou, X., He, Z., Xue, K., Wu, L., Reich, P., et al. (2016). The diversity and co-occurrence patterns of N_2-fixing communities in a CO_2-enriched grassland ecosystem. *Microb. Ecol.* 71, 604–615. doi: 10.1007/s00248-015-0659-7

Turner, B., and Joseph Wright, S. (2014). The response of microbial biomass and hydrolytic enzymes to a decade of nitrogen, phosphorus, and potassium addition in a lowland tropical rain forest. *Biogeochemistry* 117, 115–130. doi: 10.1007/s10533-013-9848-y

Verhamme, D. T., Prosser, J. I., and Nicol, G. W. (2011). Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J.* 5, 1067–1071. doi: 10.1038/ismej.2010.191

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., et al. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecol. Appl.* 7, 737–750. doi: 10.2307/2269431

Vitousek, P. M., Cassman, K. E. N., Cleveland, C., Field, C. B., Grimm, N. B., and Hole, W. (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* 57, 1–45. doi: 10.1023/A:1015798428743

Vitousek, P. M., and Hobbie, S. (2000). Heterotrophic nitrogen fixation in decomposing litter: patterns and regulation. *Ecology* 81, 2366–2376. doi: 10.1890/0012-9658(2000)081[2366:HNFIDL]2.0.CO;2

Vitousek, P. M., and Matson, P. (1988). Nitrogen transformations in a range of tropical forest soils. *Soil Biol. Biochem.* 20, 361–367. doi: 10.1016/0038-0717(88)90017-x

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. (2010). Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecol. Appl.* 20, 5–15. doi: 10.1890/08-0127.1

Walker, C. B., de la Torre, J. R., Klotz, M. G., Urakawa, H., Pinel, N., Arp, D. J., et al. (2010). *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8818–8823. doi: 10.1073/pnas.0913533107

Wallenstein, M. D., Myrold, D. D., Firestone, M., and Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecol. Appl.* 16, 2143–2152. doi: 10.1890/1051-0761(2006)016[2143:ECODCA]2.0.CO;2

Wang, F., Li, J., Wang, X., Zhang, W., Zou, B., Neher, D. A., et al. (2014). Nitrogen and phosphorus addition impact soil N_2O emission in a secondary tropical forest of South China. *Sci. Rep.* 4, 5615. doi: 10.1038/srep05615

Wang, J., Bao, J.-T., Li, X.-R., and Liu, Y.-B. (2016). Molecular ecology of *nifH* genes and transcripts along a chronosequence in revegetated areas of the Tengger desert. *Microb. Ecol.* 71, 150–163. doi: 10.1007/s00248-015-0657-9

Werner, C., Butterbach-Bahl, K., Haas, E., Hickler, T., and Kiese, R. (2007a). A global inventory of N_2O emissions from tropical rainforest soils using a detailed biogeochemical model. *Glob. Biogeochem. Cycles* 21:GB3010. doi: 10.1029/2006GB002909

Werner, C., Kiese, R., and Butterbach-Bahl, K. (2007b). Soil-atmosphere exchange of N_2O, CH4 and CO_2 and controlling environmental factors for tropical rain forest sites in western Kenya. *J. Geophys. Res. Atmos* 112:D03308. doi: 10.1029/2006JD007388

Wessén, E., Nyberg, K., Jansson, J. K., and Hallin, S. (2010). Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. *Appl. Soil Ecol.* 45, 193–200. doi: 10.1016/j.apsoil.2010.04.003

Wrage, N., Velthof, G. L., Laanbroek, H. L., and Oenema, O. (2004). "Pitfalls in measuring nitrous oxide production by nitrifiers," in *Control Nitrogen Flows Losses*, eds D. J. Hatch, D. R. Chadwick, S. C. Jarvis, and J. A. Roker (Exeter: University of Exeter), 260–267.

Wrage, N., Velthof, G. L., Van Beusichem, M. L., and Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biogeochem.* 33, 1723–1732. doi: 10.1016/S0038-0717(01)00096-7

Wright, S. J. (2013). The carbon sink in intact tropical forests. *Glob. Change Biol.* 19, 337–339. doi: 10.1111/gcb.12052

Wright, S. J., Yavitt, J. B., Wurzburger, N., Turner, B. I., Tanner, E. V. J., Sayer, E. J., et al. (2011). Potassium, phosphorus, or nitrogen limit root allocation, tree growth, or litter production in a lowland tropical forest. *Ecology* 92, 1616–1625. doi: 10.1890/10-1558.1

Wullaert, H., Homeier, J., Valarezo, C., and Wilcke, W. (2010). Response of the N and P cycles of an old-growth montane forest in Ecuador to experimental

low-level N and P amendments. *For. Ecol. Manage.* 260, 1434–1445. doi: 10.1016/j.foreco.2010.07.021

Wurzburger, N., Bellenger, J. P., Kraepiel, A. M. L., and Hedin, L. O. (2012). Molybdenum and phosphorus interact to constrain asymbiotic nitrogen fixation in tropical forests. *PLoS ONE* 7:e33710. doi: 10.1371/journal.pone.0033710

Xu, Y. B., and Cai, Z. C. (2007). Denitrification characteristics of subtropical soils in China affected by soil parent material and land use. *Eur. J. Soil Sci.* 58, 1293–1303. doi: 10.1111/j.1365-2389.2007.00923.x

Xu, Y. B., Xu, Z., Cai, Z., and Reverchon, F. (2013). Review of denitrification in tropical and subtropical soils of terrestrial ecosystems. *J. Soils Sediments* 13, 699–710. doi: 10.1007/s11368-013-0650-1

Yao, H., Campbell, C. D., Chapman, S. J., Freitag, T. E., Nicol, G. W., and Singh, B. K. (2013). Multi-factorial drivers of ammonia oxidizer communities: evidence from a national soil survey. *Environ. Microbiol.* 15, 2545–2556. doi: 10.1111/1462-2920.12141

Yao, H., Gao, Y., Nicol, G. W., Campbell, C. D., Prosser, J. I., Zhang, L., et al. (2011). Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. *Appl. Environ. Microbiol.* 77, 4618–4625. doi: 10.1128/AEM.00136-11

Yeager, C. M., Kornosky, J. L., Housman, D. C., Grote, E. E., Belnap, J., and Kuske, C. R. (2004). Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado Plateau and Chihuahuan Desert. *Appl. Environ. Microbiol.* 70, 973–983. doi: 10.1128/AEM.70.2.973-983.2004

Yeager, C. M., Northup, D. E., Grow, C. C., Barns, M., Kuske, C. R., and Barns, S. M. (2005). Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. *Appl. Environ. Microbiol.* 71, 2713–2722. doi: 10.1128/AEM.71.5.2713

Yu, Y., Zhang, J., Chen, W., Zhong, W., Zhu, T., and Cai, Z. (2014). Effect of land use on the denitrification, abundance of denitrifiers, and total nitrogen gas production in the subtropical region of China. *Biol. Fertil. Soils* 50, 105–113. doi: 10.1007/s00374-013-0839-x

Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ. Microbiol.* 5, 539–554. doi: 10.1046/j.1462-2920.2003.00451.x

Zhalnina, K., de Quadros, P. D., Camargo, F. A. O., and Triplett, E. W. (2012). Drivers of archaeal ammonia-oxidizing communities in soil. *Front. Microbiol.* 3:210. doi: 10.3389/fmicb.2012.00210

Zhang, J., Cai, Z., Cheng, Y., and Zhu, T. (2009). Denitrification and total nitrogen gas production from forest soils of Eastern China. *Soil Biol. Biochem.* 41, 2551–2557. doi: 10.1016/j.soilbio.2009.09.016

Zhang, J., Müller, C., Zhu, T., Cheng, Y., and Cai, Z. (2011). Heterotrophic nitrification is the predominant NO3- production mechanism in coniferous but not broad-leaf acid forest soil in subtropical China. *Biol. Fertil. Soils* 47, 533–542. doi: 10.1007/s00374-011-0567-z

Zhang, J., Yu, Y., Zhu, T., and Cai, Z. (2014). The mechanisms governing low denitrification capacity and high nitrogen oxide gas emissions in subtropical forest soils in China. *J. Geophys. Res. Biogeosci.* 119, 557–566. doi: 10.1002/2013JG002433

Zhang, X., Liu, W., Schloter, M., Zhang, G., Chen, Q., Huang, J., et al. (2013). Response of the abundance of key soil microbial nitrogen-cycling genes to multi-factorial global changes. *PLoS ONE* 8:e76500. doi: 10.1371/journal.pone.0076500

Zhao, W., Cai, Z., and Xu, Z. (2007). Does ammonium-based N addition influence nitrification and acidification in humid subtropical soils of China? *Plant Soil* 297, 213–221. doi: 10.1007/s11104-007-9334-1

Zhu, J., Mulder, J., Solheimslid, S. O., and Dörsch, P. (2013). Functional traits of denitrification in a subtropical forest catchment in China with high atmogenic N deposition. *Soil Biol. Biochem.* 57, 577–586. doi: 10.1016/j.soilbio.2012.09.017

Zhu, T., Meng, T., Zhang, J., Zhong, W., Muller, C., and Cai, Z. (2015). Fungi-dominant heterotrophic nitrification in a subtropical forest soil of China. *J. Soils Sediments* 15, 705–709. doi: 10.1007/s11368-014-1048-4

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616.

Zumft, W. G., and Kroneck, P. M. H. (2006). Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by bacteria and archaea. *Adv. Microb. Physiol.* 52, 107–227. doi: 10.1016/S0065-2911(06)52003-X

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Impact of Lowland Rainforest Transformation on Diversity and Composition of Soil Prokaryotic Communities in Sumatra (Indonesia)

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Prokaryotes are the most abundant and diverse group of microorganisms in soil and mediate virtually all biogeochemical cycles in terrestrial ecosystems. Thereby, they influence aboveground plant productivity and diversity. In this study, the impact of rainforest transformation to intensively managed cash crop systems on soil prokaryotic communities was investigated. The studied managed land use systems comprised rubber agroforests (jungle rubber), rubber plantations and oil palm plantations within two Indonesian landscapes Bukit Duabelas and Harapan. Soil prokaryotic community composition and diversity were assessed by pyrotag sequencing of bacterial and archaeal 16S rRNA genes. The curated dataset contained 16,413 bacterial and 1679 archaeal operational taxonomic units at species level (97% genetic identity). Analysis revealed changes in indigenous taxon-specific patterns of soil prokaryotic communities accompanying lowland rainforest transformation to jungle rubber, and intensively managed rubber and oil palm plantations. Distinct clustering of the rainforest soil communities indicated that these are different from the communities in the studied managed land use systems. The predominant bacterial taxa in all investigated soils were *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. Overall, the bacterial community shifted from proteobacterial groups in rainforest soils to *Acidobacteria* in managed soils. The archaeal soil communities were mainly represented by *Thaumarchaeota* and *Euryarchaeota*. Members of the Terrestrial Group and South African Gold Mine Group 1 (*Thaumarchaeota*) dominated in the rainforest and members of *Thermoplasmata* in the managed land use systems. The alpha and beta diversity of the soil prokaryotic communities was higher in managed land use systems than in rainforest. In the case of bacteria, this was related to soil characteristics such as pH value, exchangeable Ca and Fe content, C to N ratio, and extractable P content. Archaeal community composition

and diversity were correlated to pH value, exchangeable Fe content, water content, and total N. The distribution of bacterial and archaeal taxa involved in biological N cycle indicated functional shifts of the cycle during conversion of rainforest to plantations.

Keywords: rainforest conversion, soil microbial community composition, soil prokaryotic diversity, 16S rRNA gene, soil bacteria, soil archaea, oil palm, Sumatra

INTRODUCTION

Indonesia is one of the world's top producer and exporters of palm oil (Koh et al., 2011) and rubber (Marimin Darmawan et al., 2014). The continuous establishment of productive and profitable agricultural areas is accompanied by conversion of rainforest into highly productive agricultural land. This results in severe negative and irreversible effects on biodiversity and, thereby, on tropical ecosystem functions (Gibbs et al., 2010). Tropical rainforests are reckoned as important reservoirs of biodiversity (Gibson et al., 2011), which are threatened by anthropogenic demand for productive land.

Soil microbial communities contain the highest level of prokaryotic diversity of any environment, drive nearly all biogeochemical cycles in terrestrial ecosystems and participate in most nutrient transformations (Daniel, 2005; Falkowski et al., 2008; Delmont et al., 2012). It has been reported that land use and plant species as well as soil characteristics, such as pH, organic C content, and soil texture, shape soil microbial community composition and diversity (Nacke et al., 2011; Lauber et al., 2013; Pfeiffer et al., 2013). The conversion of rainforest to agricultural and plantation systems has a substantial impact on plant and animal diversity (Soares-Filho et al., 2006; Barnes et al., 2014). Despite the importance of soil microorganisms for ecosystem function, the response of microorganisms to land use change is poorly understood. Little is known on how environmental differences, e.g., changes in soil characteristics related to transformation of rainforests to rubber and oil palm plantations affect the composition, diversity and functions of soil microbial communities in general and at different spatial scales. Insights into drivers of microbial communities in tropical land use systems are limited, as an appropriate experimental design allowing robust statistical analysis and methods enabling fine taxonomic resolution are lacking in many studies.

The majority of available data on microbial communities have been collected in South and Central America such as Brazil, Ecuador, and Costa Rica (Carney et al., 2004; Rodrigues et al., 2013; Tischer et al., 2014). Studies targeting the impact of land use conversion on microbial community composition and diversity in tropical Asia are rare (Tripathi et al., 2012; Lee-Cruz et al., 2013). The published studies focused on microbial communities associated with deforestation and logging effects, and bacterial diversity in oil palm fruit compost (Liew et al., 2009; Tripathi et al., 2012; Lee-Cruz et al., 2013; McGuire et al., 2015). It has been suggested that deforestation for agricultural use alters microbial community composition in tropical regions (Tripathi et al., 2012, 2013; McGuire et al., 2015). DNA-based analysis of 16S rRNA genes indicated that soil bacteria of tropical forests are to some extent resilient to logging, but conversion to oil palm had a severe impact (Lee-Cruz et al., 2013). A study in the Brazilian Amazon rainforest showed that conversion of rainforest to pasture resulted in a strong response of soil microbial diversity, but in a manner different from plants and animals. In addition, a net loss of bacterial diversity was recorded (Rodrigues et al., 2013). Soil pH has been identified as the major driver of bacterial and archaeal diversity and community composition in the equatorial tropics (Tripathi et al., 2012, 2013). A recent study in Jambi (Indonesia) based on phospholipid fatty acid analysis showed that soil microbial biomass did not vary significantly between land use systems, although bacterial community structure changed (Krashevska et al., 2015). However, detailed information on composition and diversity of prokaryotic communities in these land use systems is still lacking.

The aim of this study was to assess the impact of agricultural demand-driven rainforest conversion to rubber and oil palm plantations on soil bacterial and archaeal community composition and diversity. This study was carried out in two contrasting landscapes with respect to soil fertility within the province Jambi of southwest Sumatra (Indonesia): Harapan with low-fertility loam Acrisol soil and Bukit Duabelas with relatively high-fertility clay Acrisol soil (Allen et al., 2015). In addition, we related changes in bacterial and archaeal composition and diversity to soil properties and abiotic and biotic factors. In this way, taxa specific-patterns and drivers of community composition associated with land use change were identified. We used large-scale amplicon-based pyrosequencing of 16S rRNA genes to assess prokaryotic community composition and diversity in the studied land use systems. Overall, we investigated the following hypotheses: (a) conversion of rainforests changes the distribution and abundance of dominant prokaryotic groups in soil, (b) species richness is highest in rainforest and lowest in managed plantations, (c) richness and diversity of prokaryotes are similar in rainforest and anthropogenically less-altered jungle rubber, and (d) rainforest conversion has a large-scale impact on the abundance of soil microbial groups and community composition by influencing soil properties and vice versa. Our study provides insights into the impacts of the rapidly expanding conversion of lowland rainforests to tree cash-crop plantations on prokaryotic soil communities.

MATERIALS AND METHODS

Sampling Site Description and Sample Recovery

The sampling sites are located in the province Jambi of southwest Sumatra, Indonesia (**Figure 1**, map data was obtained

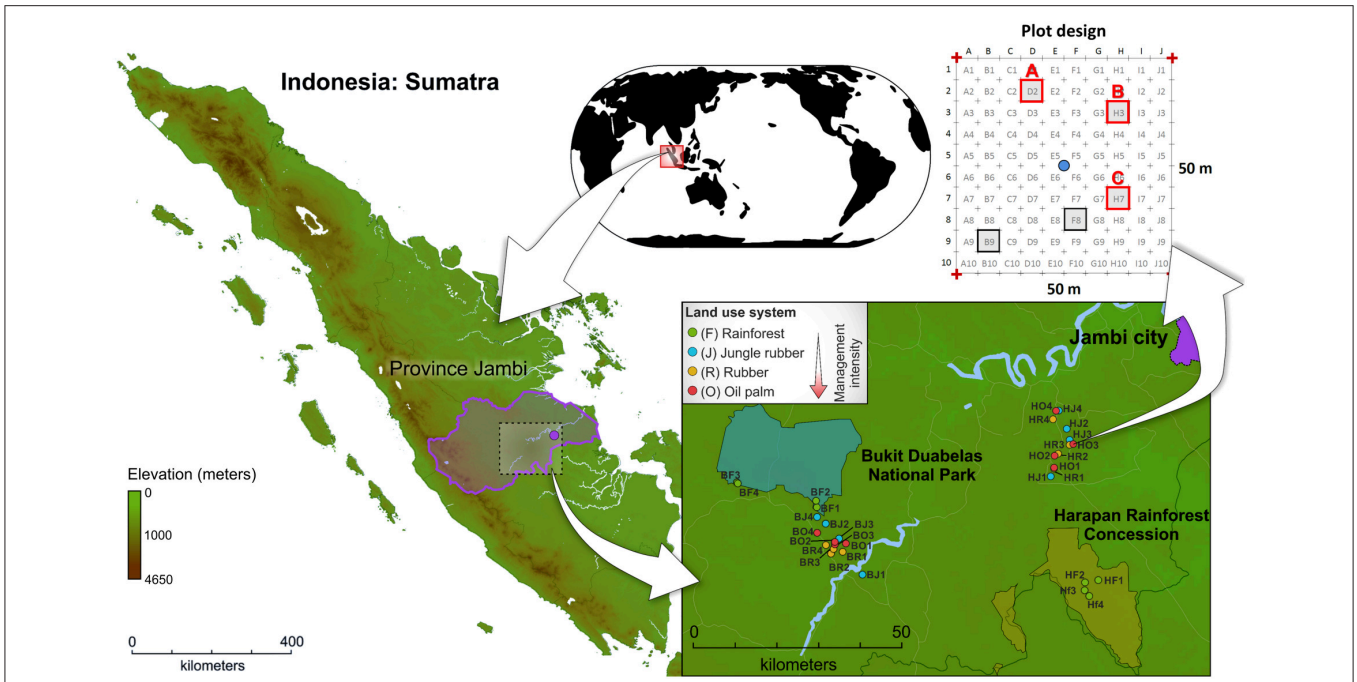

FIGURE 1 | Location of Sumatra Island (Indonesia), location of study sites within the investigated landscapes Bukit Duabelas (B) and Harapan (H), and subplot design.

from http://www.diva-gis.org/). The two landscapes, Harapan Rainforest Concession (H) and Bukit Duabelas (b), were selected for this study. Both landscapes harbor the typical land use systems in Sumatra, resulting from conversion of lowland rainforest to managed rubber and oil palm systems. In addition, suitable lowland rainforest sites (reference sites) were still present in both landscapes. Soil texture differed, with primarily loam Acrisol soils in Harapan and clay Acrisol soils in Bukit Duabelas. Within each landscape we analyzed four land use systems: secondary lowland rainforest, rubber agroforest (jungle rubber), rubber plantation, and oil palm plantation. The rainforest sites represent systems with low anthropogenic influence. Jungle rubber represents the next higher level of anthropogenic influenced land use systems. Jungle rubber is a traditional extensively managed agroforestry system, which is established by planting rubber trees into secondary rainforest. The systems with the highest anthropogenic impact are rubber and oil palm plantations, which are monocultures with high fertilizer usage and liming. The age of the rubber trees (*Hevea brasiliensis*) in jungle rubber and rubber plantation land use systems ranged from 15 to 40 and 6 to 16 years, respectively. The age of oil palm trees (*Elaeis guineensis*) in plantations varied between 8 and 15 years. The agricultural management for both plantation types included application of herbicides every 6 months and amendment of 100–300 kg ha $^{-1}$ yr $^{-1}$ inorganic NPK fertilizer in rubber plantations and 300–600 kg ha $^{-1}$ yr $^{-1}$ in oil palm plantations (for details, see Kotowska et al., 2015).

Sampling of soils was carried out from November to December 2012. The four land use systems lowland rainforest (core plots BF1-BF4 and HF1-HF4), jungle rubber (core plots BJ1-BJ4 and HJ1-HJ4), rubber plantations (core plots BR1-BR4 and HR1-HR4), and oil palm plantations (core plots BO1-BO4 and HO1-HO4) were replicated four times resulting in 32 sampling sites (for georeferences, see **Table S1**). Soil cores were recovered from three subplots of each plot, resulting in a total of 96 subplots. After removal of litter and root overlay, three soil cores (5–7 cm top soil, 10–20 g soil each) were taken with a soil corer and a shovel from each subplot at an average distance of 1.90 m to adjacent trees (random trees in rainforest). The samples were stored in sterile plastic bags. Subsequently, the soil samples were transported in cool boxes on ice packs to the laboratory in Indonesia within 12 h. The three soil samples per subplot were homogenized and coarse roots and stones (>5 mm) were removed. The composite samples were frozen and stored at a deep freezer (−40°C) until shipment to Germany. Samples were transported frozen (cool boxes and ice packs) to the German laboratory within approximately 25 h and stored there at −80°C until further use. Further information on sampling sites and experimental design are given in Barnes et al. (2014).

Nucleic Acid Isolation and Amplification of 16S rRNA Genes

To analyze the prokaryotic community richness and composition soil DNA was isolated from the four land use systems by employing the PowerSoil DNA isolation kit (Dianova, Hamburg, Germany) as recommended by the manufacturer. The hypervariable regions V3 to V5 of the 16S rRNA gene were targeted in this study. The 16S rRNA gene amplicons were generated as described by Schneider et al. (2013). In brief, we employed the forward primer

V3for_B (5′-CGTATCGCCTCCCTCGCGCCATCAG-MID-TACGGRAGGCAGCAG-3′) (Liu et al., 2007) and the reverse primer V5rev_B (5′-CTATGCGCCTTGCCAGCCCGCTCAG-MID-CCGTCAATTCMTTTGAGT-3′) (Wang and Qian, 2009) for bacteria. For amplification of archaeal 16S rRNA genes, the forward primer V3for_A (5′-CGTATCGCCTCCCTCGCGCCATCAG-MID-CCCTAYGGGGYGCASCAG-3′) (Gantner et al., 2011) and the reverse primer V5rev_A (5′-CTATGCGCCTTGCCAGCCCGCTCAG-MID-GTGCTCCCCCGCCAATTCCT-3′) (Teske and Sørensen, 2008) were used. The following thermal cycling scheme was used for amplification of partial bacterial 16S rRNA genes: initial denaturation at 98°C for 5 min, 25 cycles of denaturation at 98°C for 45 s, annealing for 45 s at 65°C, and extension at 72°C for 30 s, followed by a final extension period at 72°C for 5 min. For amplification of the archaeal 16S rRNA genes, the annealing temperature was adjusted to 60°C. For two subplots (BF4 and HF3) we were unable to generate archaeal amplicons. All amplicon PCRs were performed in triplicate and pooled in equimolar amounts for sequencing. The Göttingen Genomics Laboratory determined the sequences of the 16S rRNA gene amplicons by using a 454 GS-FLX sequencer (Roche, Mannheim, Germany) and Titanium chemistry following the instructions of the manufacturer for amplicon sequencing.

Bioinformatic Analysis of 16S rRNA Gene Sequences

The resulting 16S rRNA gene sequences were processed and analyzed employing QIIME 1.8 (Caporaso et al., 2010). Initially, sequences shorter than 300 bp, containing unresolved nucleotides, exhibiting an average quality score lower than 25, harbor mismatches longer than 3 bp in the forward primer, or possessing homopolymers longer than 8 bp were removed with *split_libraries.py*. Additionally, we used cutadapt (Martin, 2011) with default settings for efficient reverse primer removal. Subsequently, pyrosequencing noise was removed by employing Acacia (Bragg et al., 2012) with default settings. Chimeric sequences were removed using UCHIME (Edgar et al., 2011) with Ribosomal Database Project (RDP) (Cole et al., 2014) as reference dataset (trainset10_082014_rmdup.fasta).

Operational taxonomic unit (OTU) determination was performed at a genetic divergence of 3% (species level) with *pick_open_reference_otus.py* using the Silva NR SSU 119 database as reference (Quast et al., 2013). Taxonomic classification was performed with *parallel_assign_taxonomy_blast.py* against the same database. OTU tables were created using *make_otu_table.py*. Singletons, chloroplasts, unclassified OTUs and extrinsic domain OTUs were removed from the table by employing *filter_otu_table.py*. Singletons were removed to improve comparability and avoid possible inclusion of artificial sequences (Zhou et al., 2011). Sample comparisons were performed at the same surveying effort (bacteria 6800 and archaea 2000 sequences). Diversity estimates and rarefaction curves were generated by employing *alpha_rarefaction.py*. Non-metric multidimensional scaling (NMDS) and statistical tests were performed with the vegan package (Oksanen et al., 2015) in R (R Development Core Team, 2013) and based on weighted Unifrac (Lozupone et al., 2011) distance matrixes. Significance was determined using the *envfit* function of vegan package in R (Gergs and Rothhaupt, 2015) to fit environmental vectors and factors onto the NMDS. Significance of tested variables are indicated in brackets. Profile clustering networks were constructed based on complete and subsampled OTU tables using the QIIME script *make_otu_network.py*.

Soil Characteristics

Soil parameters and properties, i.e., pH, P, N, C, C to N ratio, Al, Ca, Fe, K, Mg, Mn, Na, effective cation exchange capacity (ECEC) and base saturation for all analyzed samples were retrieved from Allen et al. (2015). Furthermore, basal respiration, microbial biomass, and soil moisture were retrieved from Krashevska et al. (2015). These data were used for statistical tests as detailed in **Table S1**. Data was tested for normal distribution with *shapiro.test* of stats package in R (R Development Core Team, 2013). Data that did not pass normality test ($P < 0.05$) was log transformed and normality test was repeated. Only data that passed normality test was used for further analyses. ANOVA analyses were performed with the *aov* function of stats package in R (R Development Core Team, 2013). Comparisons of land use soil characteristics were performed with Tukey's HSD (Honestly Significant Difference) by using *HSD.test* function of agricolae package in R (Mendiburu, 2015; **Table S2**).

Accession Numbers

The 16S rRNA gene sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number SRP056374.

RESULTS AND DISCUSSION

Study Site and General Soil Characteristics

The study formed part of the "Ecological and Socioeconomic Functions of Tropical Lowland Rainforest Transformation Systems" (EFForTS) collaborative research center, which analyzes various aspects of tropical lowland rainforest conversion to agricultural systems in Indonesia, including the impact on aboveground and belowground biodiversity, soil fertility, nutrient fluxes and greenhouse gas emissions as well as the economic, social, cultural and political dimensions (Barnes et al., 2014; Guillaume et al., 2015; Krashevska et al., 2015). We analyzed an agricultural management gradient with increasing intensity from jungle rubber over rubber plantations to oil palm plantations in two landscapes (Bukit Duabelas and Harapan). Soils from lowland rainforest sites served as reference. The soils comprised relatively fertile, clay loam Acrisol soil in Bukit Duabelas and less fertile, loam Acrisol soil in Harapan (**Table S1**).

Although the investigated systems were non-artificial, the soil parameters showed clear patterns for the land use systems (**Table S1** and **Figure S1**). The analyses of soil characteristics between land use systems by ANOVA and Tukey's HSD showed that the soils of the analyzed land use types did not vary significantly in N, C, basal respiration, microbial biomass, moisture and silt content (**Table S2**). Significant differences

between land use types were observed for pH values, P content and clay content (**Table S2**). Soil pH increased slightly from an average of 4.21 to 4.45 from rainforest to oil palm plantations in both landscapes, which likely is due to liming. Bioavailable micro- and macroelements, i.e., Mn, Na, C, Ca, Fe, Mg, and N had an overall higher concentration in Bukit Duabelas soils than in Harapan soils (see **Table S1** and **Figure S2**). Organic carbon was generally lower in the managed systems rubber and oil palm. Soil moisture was roughly three-fold higher in Bukit Duabelas than in Harapan (**Table S1**).

Effect of Rainforest Transformation on Bacterial Diversity and Community Composition

DNA from each subplot was used for amplification of the V3–V5 hypervariable region of the bacterial 16S rRNA gene. Sequencing and quality filtering resulted in 1,367,923 high-quality 16S rRNA gene sequences from all subplots. After removal of singletons, the dataset comprised 16,413 OTUs at 97% genetic identity. After subsampling (6800 sequences per sample), the average number of OTUs per subplot was 1160 ± 245 ranging from 604 (BF4b) to 1825 (HO2b) OTUs (**Table S3**).

Soil bacterial diversity significantly responded to land use change from rainforest to plantations ($P = 0.001$, $r^2 = 0.7875$). Richness and diversity incrementally rose with increasing management intensity from rainforest to oil palm plantations (rainforest < jungle rubber < rubber plantations < oil palm plantations, see **Figure S3**). This was different from the responses of animals and plant diversity to land use conversion, which showed the opposite trend (Barnes et al., 2014). Accordingly, Shannon indices of diversity of the bacterial communities differed between rainforest in relation to the managed land use systems ($P = 0.001$, $r^2 = 0.5956$, **Table S3**). Phylogenetic diversity (PD, $P = 0.001$, $r^2 = 0.7349$) showed a similar trend, indicating highest diversity in the managed land use systems (**Table S3**). Rarefaction curves showed slight saturation at the same surveying effort, which indicates that the datasets covered all main bacterial groups thriving in the investigated land use systems (**Figure S3**). In addition, the bacterial diversity slightly varied between the two studied landscapes. In all land use systems the diversity was slightly higher in the Harapan than in the Bukit Duabelas region. Landscape alone had no significant effect on bacterial community composition ($P > 0.8$).

The analysis of the bacterial community composition and abundance of taxa within the different land use systems revealed the main bacterial groups thriving in the studied systems and their different abundances (**Figure 2**). The composition of soil bacterial communities varied between the different land use systems, but was very similar within a land use system. The most abundant phyla in all samples were *Acidobacteria* (42.7%) followed by *Proteobacteria* (37.7%), *Actinobacteria* (12.6%), and to a lesser degree Candidate Division WD272 (1.6%), *Firmicutes* (1.2%), *Chloroflexi* (1.0%), *Nitrospirae* (0.9%), *Gemmatimonadetes* (0.8%), and *Bacteroidetes* (0.6%). These bacterial phyla and Candidate Division are common in a variety of different soils, including non-tropical forest, grassland and agricultural soils (da C Jesus et al., 2009; Nacke et al., 2011; Lauber et al., 2013; Tripathi et al., 2014).

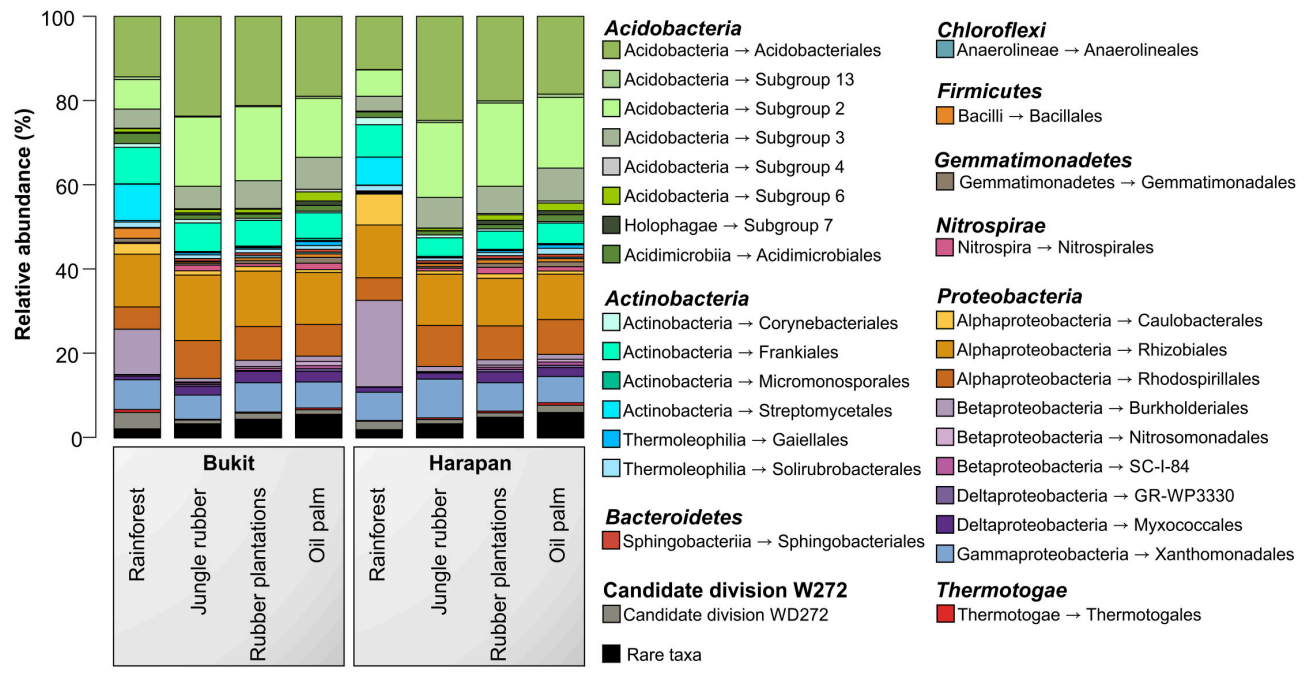

FIGURE 2 | Bacterial community composition based on relative abundances separated by landscape and land use system. The results of all analyzed samples of each land use system in a landscape were summarized (for individual results, see bacterial OTU table **Data Sheet 1**).

Soil bacterial communities in rainforest sites of both studied landscapes were very similar (**Figure 2**). The same was found for the communities in the other land use systems of both landscapes, indicating a management-specific shift of the bacterial community structure. Differences between the studied land use systems were mainly encountered at higher taxonomic resolution (**Figure 3**).

A comparison of *Acidobacteria* at order level revealed that the relative abundances of subgroups 2, 4, 5, 6, and 7 were higher in soils of the managed systems than in rainforest soils. *Acidobacteriales* showed the highest abundance in jungle rubber, followed by rubber, oil palm plantations, and rainforest. Additionally, our results indicate a general pH optimum for most of the encountered acidobacterial groups at a pH of approximately 4.4 (data not shown). Within the *Actinobacteria*, *Acidimicrobiales* exhibited significant higher relative abundances in rainforest and oil palm than in jungle rubber and rubber plantations. *Frankiales*, *Streptomycetales*, and *Corynebacteriales* were more abundant in rainforests than in the managed land use systems.

Among *Alphaproteobacteria*, *Rhizobiales* were abundant in all land use systems, whereas *Caulobacterales* revealed a higher abundance in rainforest than in the managed land use systems. *Rhodospirillales* showed the opposite trend. The betaproteobacterial *Burkoholderiales* (i.e., *Burkholderia tropica*; Reis et al., 2004) were more abundant in rainforest (up to 20.5% in Harapan rainforest soils) compared to the managed systems (<1.5%). *Burkholderia* seems to be one of the key bacterial groups for nitrogen-fixation in rainforest soils, since several species are known as plant-associated nitrogen-fixing bacteria (Estrada-De Los Santos et al., 2001). The decrease of *Burkholderia* species followed the increased use of fertilizer in the plantation systems. The increase of bioavailable nitrogen through fertilization in intensively managed soils provides other bacterial taxa with improved growth conditions. This leads to a decrease of nitrogen-fixing bacteria. This observation is confirmed by

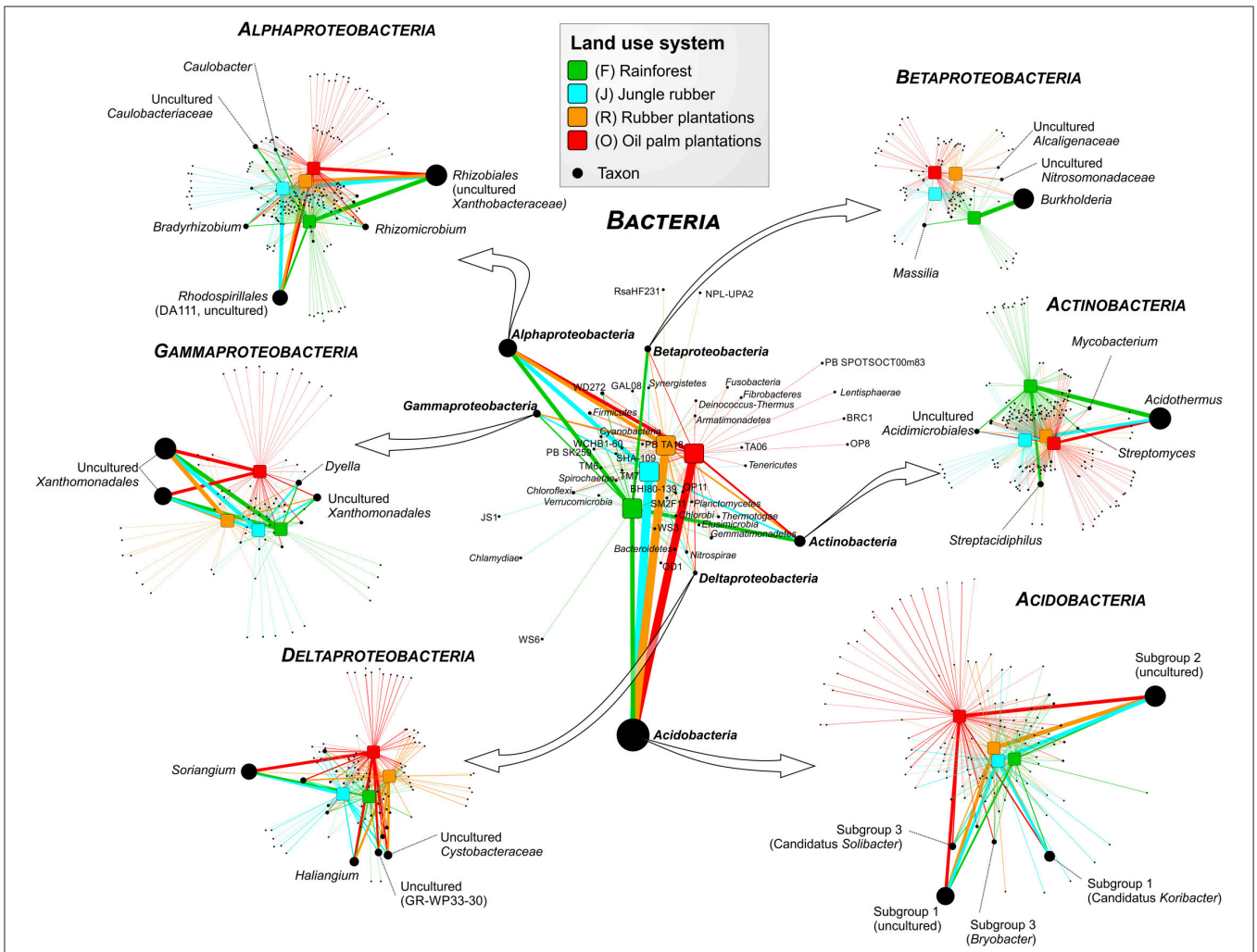

FIGURE 3 | Profile clustering network analysis of bacterial phyla and proteobacterial classes in the land use systems and detailed networks of the most abundant classes. The width of the node connecting lines defines the mean relative abundance of the bacterial taxon in the corresponding land use system. The size of each node is proportional to the mean taxon abundance between all land use systems.

studies on N availability in the investigated land use systems, which decreased along the described land use gradient (Allen et al., 2015). Members of the *Nitrosomonadales* and SC-I-84 were almost absent in rainforest soils, but showed an increasing abundance (up to 1%) along the gradient from unmanaged rainforest to intensively managed oil palm plantations. The increased abundance of Nitrosomonadales, which are known as ammonia-oxidizing bacteria (Shen et al., 2012), also followed the increase of fertilizer treatment in the intensively managed plantations.

The main representatives of the *Deltaproteobacteria* were the fungi-like *Myxococcales* (*Soriangium* and *Haliangium*) and GR-WP33-30, which were slightly more abundant in the managed soils. The increased abundance of myxobacteria in managed systems is another indication of increased anthropogenic influence (fertilization, dung) within managed land use systems (Brenner et al., 2005). Within the *Gammaproteobacteria*, the *Xanthomonadales* were the predominant order, however, their abundance did not differ significantly between the analyzed land use systems. Interestingly, conversion to fertilized soils in plantations did not increase the abundance of potentially harmful *Gammaproteobacteria* in soils, as recently shown for Mexican agricultural soils fertilized with wastewater (Broszat et al., 2014).

Despite the higher diversity in the managed land use systems, several phylogenetic groups were specific for the rainforest soils and not present in other soils, including several genera within the *Alphaproteobacteria* and *Actinobacteria*. Thus, conversion of rainforests leads to a loss of rainforest endemic bacterial groups. However, several taxa thrive only in managed soils, which is likely due to higher pH and higher nutrient availability derived from fertilization. Other effects can also be accounted for the occurrence of certain taxa, i.e., the abundance of photosynthetic *Cyanobacteria* and *Rhodospirillales* increased in oil palm plantations. This is presumably due to the more open canopy (monocultures) in oil palm plantations compared to rainforest, which results in higher light levels on the ground supporting growth of photosynthetically active bacterial groups. Other examples were members of the *Bacteroidetes*, i.e., uncultured *Chitinophagaceae*, which were slightly more abundant in managed systems. Members of this family are known as chitin degraders and the higher abundance might be linked to an increase in fungal abundance in managed systems (jungle rubber and rubber plantations), as shown by Krashevska et al. (2015).

Influence of Soil Attributes on Prokaryotic Communities

NMDS confirmed that the dissimilarities in community composition were driven by conversion of rainforest to agriculturally managed systems (**Figure 4A**). Soil bacterial communities in managed systems such as rubber and oil palm plantations clearly separated from those of rainforest soils. The rainforest communities formed a distinct cluster. From all measured biotic and abiotic soil parameters, environmental parameters, such as soil pH and base saturation (pH, $P = 0.001$, $r^2 = 0.4465$; base saturation, $P = 0.001$, $r^2 = 0.4573$), and to a lesser extent exchangeable Ca and Fe content, C to N ratio, and extractable P content correlated with bacterial community

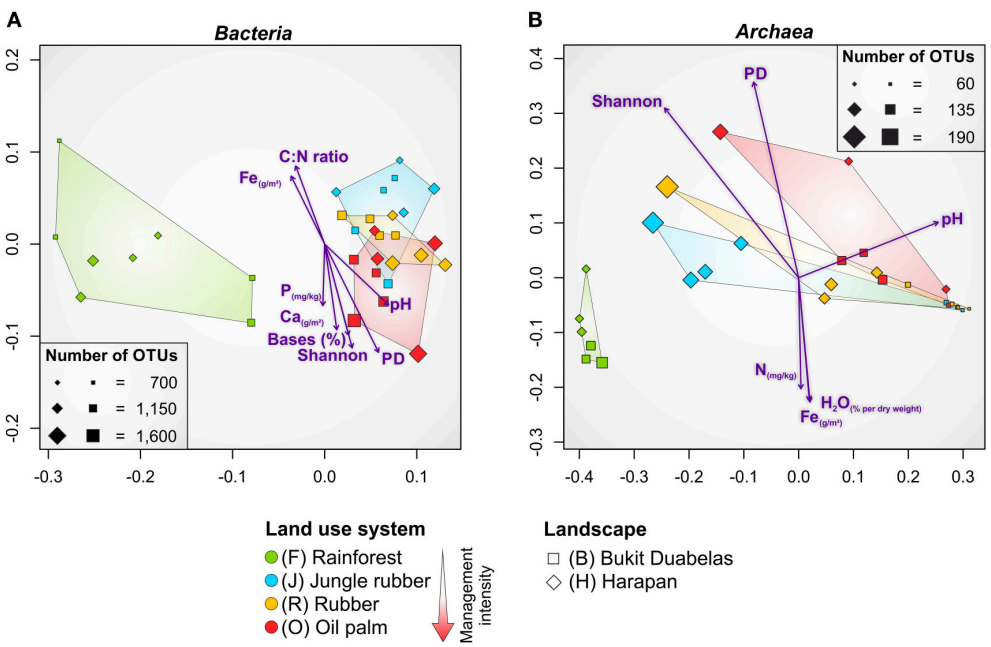

FIGURE 4 | Non-metric multidimensional scaling (NMDS) of bacterial (A) and archaeal (B) community composition in all core plots of the land use systems rainforest, jungle rubber, rubber and oil palm based on weighted Unifrac (Lozupone et al., 2011) distance matrices. Significant correlations of environmental parameters and diversity metrices (Shannon, PD) to community composition are shown by purple arrows ($P \leq 0.045$). Size of plots (squares and diamonds) corresponds to number of observed OTUs at species level (97% genetic similarity). Bases, base saturation; PD, phylogenetic diversity.

composition (Ca, $P = 0.003$, $r^2 = 0.3861$; Fe, $P = 0.011$, $r^2 = 0.2957$; C to N ratio, $P = 0.003$, $r^2 = 0.3078$; P, $P = 0.042$, $r^2 = 0.1801$). It is known that soil pH is one of the major drivers of bacterial community structure (Lauber et al., 2009; Rousk et al., 2010; Nacke et al., 2011), but in the here analyzed landscapes base saturation exhibited a higher impact. Since base saturation is closely connected to soil pH, this effect was expected. The exchangeable Ca content generally increased from rainforest to oil palm plantation due to liming. The C to N ratio, a predictor for nitrogen availability, was lower in managed soils due to fertilizer usage. In addition to plants, also specialized bacterial taxa use the supplied nitrogen fertilizer. This was indicated by the increase in ammonia-oxidizing bacteria (*Nitrosomonadales*) in plantations. Exchangeable Fe was higher in rainforest soils possibly due to the higher acidity. Thus, fertilization temporarily increased bacterial diversity, however, recovery potential of managed soils was not investigated in this study. To analyze the recovery potential of agriculturally used rainforest soils the absence of fertilization for longer time periods would be a requirement.

As recorded for bacterial communities, NMDS analysis of the soil archaeal community composition also showed that the dissimilarities were driven by conversion of rainforest to managed systems (**Figure 4B**). The soil archaeal communities in managed land use systems also clearly separated from that in rainforest soils and showed distinct clustering. Soil pH was also related to archaeal community structure but compared to bacteria to a lesser extent (pH, $P = 0.002$, $r^2 = 0.3768$). Additionally, less significant association of archaeal communities was observed for Fe content ($P = 0.020$, $r^2 = 0.2599$), water content ($P = 0.029$, $r^2 = 0.2526$), and total N ($P = 0.034$, $r^2 = 0.2058$). This suggests a negative correlation of archaeal taxa with an increase of soil moisture, Fe and N content, indicating the preference of certain archaea for habitats with harsher (more extreme) conditions such as low pH values, low water content, and limited availability of nutrients and energy sources (Chaban et al., 2006).

Effect of Rainforest Transformation on Archaeal Diversity and Community Composition

Archaea are important members of soil prokaryotic communities, but constitute on average only about 2% of the prokaryotic soil community (Bates et al., 2011). The entire curated dataset of all analyzed plots contained 438,500 archaeal 16S rRNA gene sequences and comprised 1679 OTUs at species level (97% genetic identity). The average number of OTUs per sample was 113 ± 41 and ranged from 53 (BR3a) to 234 (HJ2a) OTUs (**Table S4**). Archaeal diversity was generally higher in the Harapan region compared to the corresponding land use systems of the Bukit Duabelas region. The archaeal communities of Harapan soils followed roughly the same trend as the bacterial communities and diversity increased in the managed systems (**Figure S4** and **Table S4**). The communities in the soils from the Bukit Duabelas region revealed a different behavior, as the archaeal communities in both rubber land use systems showed a lower diversity compared to rainforest and oil palm plantations. This difference might be linked to the lower humidity and reduced availability of nutrients and energy sources in the Harapan soils (see **Figure 4B** and **Table S1**).

The most abundant archaeal phyla in all samples were *Thaumarchaeota* (54.6%) and *Euryarchaeota* (45.3%). *Crenarchaeota* were present in all land use systems but only in very low amounts (<0.1%). Archaeal diversity varied among landscapes and land use systems. A distinct shift from *Thaumarchaeota* to *Euryarchaeota* was observed from rainforest to the managed land use system in both landscapes. This effect was more pronounced in the Bukit Duabelas landscape (**Figure 5**).

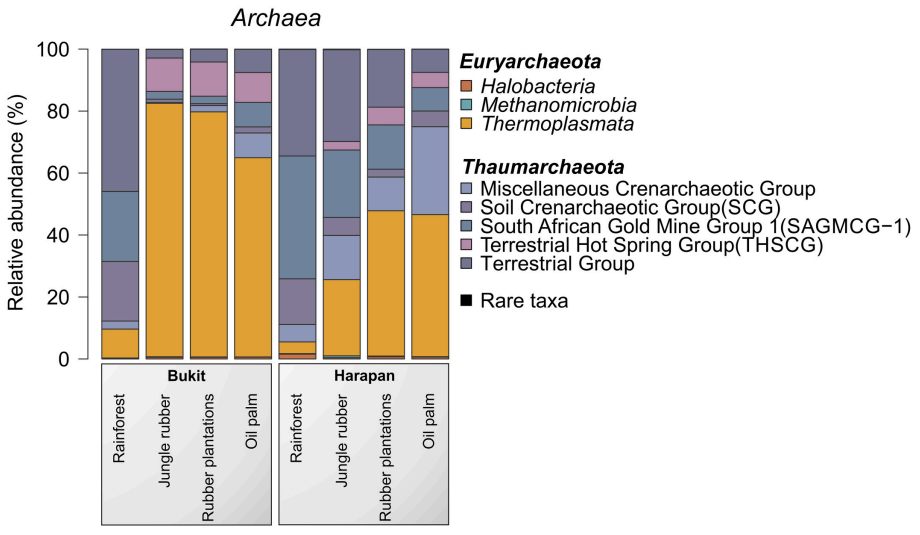

FIGURE 5 | Archaeal community composition based on relative abundances separated by landscape and land use system. The results of all analyzed samples of a land use system in a landscape were summarized (for individual results, see archaeal OTU table **Data Sheet 2**).

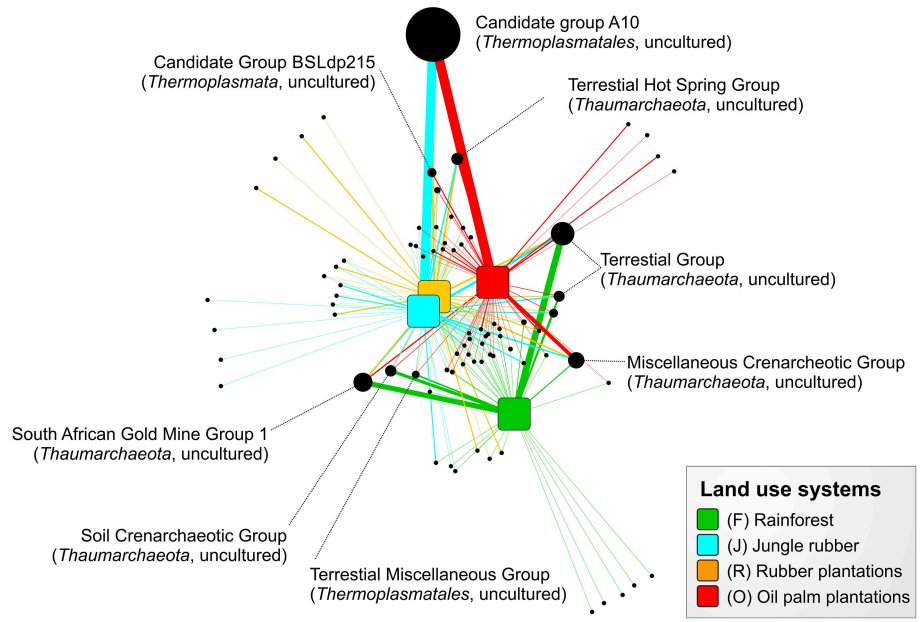

FIGURE 6 | Profile clustering network analysis of the archaeal taxa composition in the land use systems. The width of the node connecting lines defines the mean relative abundance of the archaeal taxon in the according land use system. The size of each node is proportional to the mean taxon abundance between all land use systems.

Thaumarchaeota contain important taxa involved in the soil N cycle such as *Nitrososphaera* species. *Nitrososphaera* species have been recently isolated and characterized. Members of these species are involved in nitrification by performing oxidation of ammonia (Spang et al., 2012). The abundance of uncultured members of Candidatus *Nitrososphaera*, which is part of Soil Crenarchaeotic Group (SCG), increased almost five-fold from rainforest to oil palm (0.2–0.9%). Thus, the abundance of nitrogen-oxidizing *Thaumarchaeota* rose with increasing fertilization performed in the managed plantations (rubber and oil palm plantations). This trend was coupled with a concurrent increase in ammonia-oxidizing bacteria (*Nitrosomonadales*, see above). This suggested interactions between these groups and functional changes in the biological N cycle during transformation of rainforest into plantations (Bates et al., 2011).

For the Bukit Duabelas landscape soils, the *Thaumarchaeota* and *Euryarchaeota* showed distinct differences in relative abundances between rainforest and the other land use systems (**Figure 6**). A comparison of *Thaumarchaeota* at order level revealed that the relative abundance of the Terrestrial Group was higher in rainforest than in managed soils. This also applied for subgroups SAGMGC-1 and SCG whereas Terrestrial Hot Spring Group (THSCG) and Miscellanous Crenarchaeotic Group (MCG) increased in managed soils. Members of SAGMGC-1 include ammonia-oxidizing archaea and prefer low pH environments (Auguet and Casamayor, 2013). This was also observed for Harapan soils, but in comparison to Bukit Duabelas soils the change from *Thaumarchaeota* to *Euryarchaeota* was less pronounced. Additionally, the THSCG only slightly increased in abundance from rainforest to oil palm. The MCG was more abundant in Harapan than in Bukit Duabelas soils. The predominant *Thermoplasmatales* was Candidate Group A10, which was absent from rainforest soils. Interestingly, the dominant OTUs of this group have relatives in extreme environments like sediments of thermoacidophilic volcanic springs (Eme et al., 2013; Wemheuer et al., 2013), indicating that the effect of pH outweighed the influence of temperature. Unfortunately, little is known on the metabolic potential of the other archaeal taxa and possible traits cannot be deduced currently.

CONCLUSION

According to our hypothesis (a), the conversion of rainforest to managed systems significantly impacts soil prokaryotic (bacteria and archaea) community structure, diversity and correspondingly, functional traits. Distinct clustering of the rainforest soil communities indicated that these are different from the communities in the studied managed land use systems.

The soil communities in the low-intensity managed jungle rubber system were more closely related to that in the plantation systems than to that in rainforest. This is in contrast to our hypothesis (c), that prokaryotes in rainforest and jungle rubber sites are more similar. However, jungle rubber sites are considered as the intermediate system between rainforest and

plantation sites. Additionally, the jungle rubber soil communities showed greater similarity to those in rubber plantations than to those in rainforest soils, indicating a management and possibly tree species impact.

We recorded an increase of soil prokaryotic diversity from rainforest to oil palm plantations. This is in contrast to animals, fungi, and plants and our hypothesis (b) stating that rainforest conversion to agriculturally managed systems negatively impacts prokaryotic diversity. Several bacterial and archaeal taxa were specific for rainforest soils and not present in the other land use systems. Thus, despite an increase in diversity in the managed systems the conversion of rainforests to managed systems leads to a net loss in prokaryotic biodiversity, which is coupled to a loss of traits (Rodrigues et al., 2013). The long-term effect of this loss is not known and has to be determined in long-term studies, e.g., analysis of the recovery potential of soil prokaryotic communities after reforestation or in the absence of management treatments like fertilization. In particular, it is unknown whether the rainforest endemic taxa are truly locally extinct or still present in the managed land use systems, but in such low abundance that they were not detected by our surveying effort. However, saturation of the rarefaction curves indicated that we provided a comprehensive survey of soil prokaryotic communities in the studied systems. Nevertheless, we also observed unique prokaryotic taxa in the other land use systems, resulting in highest diversity in oil palm plantations. It will be awarding to evaluate if traits from endemic species exist in other taxa that are present in the soil communities of other land use systems, as redundancy for many biogeochemical and other gene families across soil microbial groups exists.

In accordance with our hypothesis (d), the conversion of rainforests resulted in significant changes of the prokaryotic community composition. The reduction of nitrogen-fixing bacterial community members in plantations due to fertilizer usage may negatively impact soil fertility on the long term. In addition, it is indicated that treatment-induced changes of soil characteristics, especially vigorous fertilization in oil palm plantations, support prokaryotic diversity.

In the near future, we will focus on the analysis of temporal and treatment-induced (e.g., pesticide treatment) changes of the soil microbial community structures and their functions along the different land use systems in the tropics. In addition, interaction networks between different prokaryotic functional groups and other soil organisms including fungi will be investigated to deepen our understanding of global impacts of large-scale rainforest transformation on soil ecosystem functions.

AUTHOR CONTRIBUTIONS

RD designed and conceived the study; Soil sampling for prokaryotic community analysis was performed by ME, MW, and AM; DS, ME, KA, SK, VK, and MH carried out the field and laboratory work; DS, KA, and VK prepared and analyzed the data; all authors interpreted the results and wrote the paper.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01339

Data Sheet 1 | Bacterial OTU table at subplot level including taxonomic information.

Data Sheet 2 | Archaeal OTU table at subplot level including taxonomic information.

Table S1 | Soil characteristics and georeferences of the analyzed plots.

Table S2 | Statistical analyses of soil characteristics.

Table S3 | Observed bacterial OTUs and diversity at subplot level with summaries at plot level.

Table S4 | Observed archaeal OTUs and diversity at subplot level with summaries at plot level.

Figure S1 | Box plots of soil characteristics summarized by land use system.

Figure S2 | Box plots of soil characteristics summarized by landscape and land use system.

Figure S3 | Rarefaction analyses of the bacterial diversity of the two analyzed landscapes and four land use systems.

Figure S4 | Rarefaction analyses of the archaeal diversity of the two analyzed landscapes and four land use systems.

REFERENCES

Allen, K., Corre, M. D., Tjoa, A., and Veldkamp, E. (2015). Soil nitrogen-cycling responses to conversion of lowland forests to oil palm and rubber plantations in Sumatra, Indonesia. *PLoS ONE* 10:e0133325. doi: 10.1371/journal.pone.0133325

Auguet, J. C., and Casamayor, E. O. (2013). Partitioning of *Thaumarchaeota* populations along environmental gradients in high mountain lakes. *FEMS Microbiol. Ecol.* 84, 154–164. doi: 10.1111/1574-6941.12047

Barnes, A. D., Jochum, M., Mumme, S., Haneda, N. F., Farajallah, A., Widarto, T. H., et al. (2014). Consequences of tropical land use for multitrophic biodiversity and ecosystem functioning. *Nat. Commun.* 5:5351. doi: 10.1038/ncomms6351

Bates, S. T., Berg-Lyons, D., Caporaso, J. G., Walters, W. A., Knight, R., and Fierer, N. (2011). Examining the global distribution of dominant archaeal populations in soil. *ISME J.* 5, 908–917. doi: 10.1038/ismej.2010.171

Bragg, L., Stone, G., Imelfort, M., Hugenholtz, P., and Tyson, G. W. (2012). Fast, accurate error-correction of amplicon pyrosequences using Acacia. *Nat. Methods* 9, 425–426. doi: 10.1038/nmeth.1990

Brenner, D. J., Krieg, N. R., Staley, J. T., and Garrity, G. M. (2005). *Bergey's Manual of Systematic Bacteriology*, Vol. 2, Part C. New York, NY: Springer.

Broszat, M., Nacke, H., Blasi, R., Siebe, C., Huebner, J., Daniel, R., et al. (2014). Wastewater irrigation increases the abundance of potentially harmful *Gammaproteobacteria* in soils in Mezquital Valley, Mexico. *Appl. Environ. Microbiol.* 80, 5282–5291. doi: 10.1128/AEM.01295-14

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carney, K. M., Matson, P. A., and Bohannan, B. J. M. (2004). Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. *Ecol. Lett.* 7, 684–694. doi: 10.1111/j.1461-0248.2004.00628.x

Chaban, B., Ng, S. Y., and Jarrell, K. F. (2006). Archaeal habitats–from the extreme to the ordinary. *Can. J. Microbiol.* 52, 73–116. doi: 10.1139/w05-147

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. doi: 10.1093/nar/gkt1244

da C Jesus, E., Marsh, T. L., Tiedje, J. M., and de S Moreira, F. M. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Daniel, R. (2005). The metagenomics of soil. *Nat. Rev. Microbiol.* 3, 470–478. doi: 10.1038/nrmicro1160

Delmont, T. O., Prestat, E., Keegan, K. P., Faubladier, M., Robe, P., Clark, I. M., et al. (2012). Structure, fluctuation and magnitude of a natural grassland soil metagenome. *ISME J.* 6, 1677–1687. doi: 10.1038/ismej.2011.197

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Eme, L., Reigstad, L. J., Spang, A., Lanzén, A., Weinmaier, T., Rattei, T., et al. (2013). Metagenomics of Kamchatkan hot spring filaments reveal two new major (hyper)thermophilic lineages related to *Thaumarchaeota*. *Res. Microbiol.* 164, 425–438. doi: 10.1016/j.resmic.2013.02.006

Estrada-De Los Santos, P., Bustillos-Cristales, R., and Caballero-Mellado, J. (2001). Burkholderia, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl. Environ. Microbiol.* 67, 2790–2798. doi: 10.1128/AEM.67.6.2790-2798.2001

Falkowski, P. G., Fenchel, T., and Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* 320, 1034–1039. doi: 10.1126/science.1153213

Gantner, S., Andersson, A. F., Alonso-Sáez, L., and Bertilsson, S. (2011). Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. *J. Microbiol. Methods* 84, 12–18. doi: 10.1016/j.mimet.2010.10.001

Gergs, R., and Rothhaupt, K.-O. (2015). Invasive species as driving factors for the structure of benthic communities in Lake Constance, Germany. *Hydrobiologia* 746, 245–254. doi: 10.1007/s10750-014-1931-4

Gibbs, H. K., Ruesch, A. S., Achard, F., Clayton, M. K., Holmgren, P., Ramankutty, N., et al. (2010). Tropical forests were the primary sources of new agricultural land in the 1980s and 1990s. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16732–16737. doi: 10.1073/pnas.0910275107

Gibson, L., Lee, T. M., Koh, L. P., Brook, B. W., Gardner, T. A., Barlow, J., et al. (2011). Primary forests are irreplaceable for sustaining tropical biodiversity. *Nature* 478, 378–381. doi: 10.1038/nature10425

Guillaume, T., Damris, M., and Kuzyakov, Y. (2015). Losses of soil carbon by converting tropical forest to plantations: erosion and decomposition estimated by delta C. *Glob. Chang Biol.* 21, 3548–3560. doi: 10.1111/gcb.12907

Koh, L. P., Miettinen, J., Liew, S. C., and Ghazoul, J. (2011). Remotely sensed evidence of tropical peatland conversion to oil palm. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5127–5132. doi: 10.1073/pnas.1018776108

Kotowska, M. M., Leuschner, C., Triadiati, T., Meriem, S., and Hertel, D. (2015). Quantifying above- and belowground biomass carbon loss with forest conversion in tropical lowlands of Sumatra (Indonesia). *Glob. Chang. Biol.* 21, 3620–3634. doi: 10.1111/gcb.12979

Krashevska, V., Klarner, B., Widyastuti, R., Maraun, M., and Scheu, S. (2015). Impact of tropical lowland rainforest conversion into rubber and oil palm plantations on soil microbial communities. *Biol. Fertil. Soils* 51, 697–705. doi: 10.1007/s00374-015-1021-4

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi: 10.1128/AEM.00335-09

Lauber, C. L., Ramirez, K. S., Aanderud, Z., Lennon, J., and Fierer, N. (2013). Temporal variability in soil microbial communities across land-use types. *ISME J.* 7, 1641–1650. doi: 10.1038/ismej.2013.50

Lee-Cruz, L., Edwards, D. P., Tripathi, B. M., and Adams, J. M. (2013). Impact of logging and forest conversion to oil palm plantations on soil bacterial communities in Borneo. *Appl. Environ. Microbiol.* 79, 7290–7297. doi: 10.1128/AEM.02541-13

Liew, P. W., Jong, B. C., Goh, C. M., and Ahmad, M. (2009). Bacterial diversity associated with empty oil palm fruit bunch compost as revealed by cultivation-independent analyses of PCR-amplified 16S rRNA genes. *J. Gen. Appl. Microbiol.* 55, 233–240. doi: 10.2323/jgam.55.233

Liu, Z., Lozupone, C., Hamady, M., Bushman, F. D., and Knight, R. (2007). Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Res.* 35, e120. doi: 10.1093/nar/gkm541

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *ISME J.* 5, 169–172. doi: 10.1038/ismej.2010.133

Marimin Darmawan, M. A., Machfud Islam Fajar Putra M. P., and Wiguna, B. (2014). Value chain analysis for green productivity improvement in the natural rubber supply chain: a case study. *J. Clean. Prod.* 85, 201–211. doi: 10.1016/j.jclepro.2014.01.098

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* 17, 10–12. doi: 10.14806/ej.17.1.200

McGuire, K. L., D'Angelo, H., Brearley, F. Q., Gedallovich, S. M., Babar, N., Yang, N., et al. (2015). Responses of soil fungi to logging and oil palm agriculture in southeast asian tropical forests. *Microb. Ecol.* 69, 733–747. doi: 10.1007/s00248-014-0468-4

Mendiburu, F. D. (2015). *agricolae: Statistical Procedures for Agricultural Research*. R Package Version 1.2-3. Available online at: https://cran.r-project.org/web/packages/agricolae/index.html

Nacke, H., Thurmer, A., Wollherr, A., Will, C., Hodac, L., Herold, N., et al. (2011). Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils. *PLoS ONE* 6:e17000. doi: 10.1371/journal.pone.0017000

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2015). *vegan: Community Ecology Package*. R Package Version 2.2-1. Available online at: https://cran.r-project.org/web/packages/vegan/index.html

Pfeiffer, B., Fender, A.-C., Lasota, S., Hertel, D., Jungkunst, H. F., and Daniel, R. (2013). Leaf litter is the main driver for changes in bacterial community structures in the rhizosphere of ash and beech. *Agric. Ecosyst. Environ. Appl. Soil Ecol.* 72, 150–160. doi: 10.1016/j.apsoil.2013.06.008

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

R Development Core Team (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org

Reis, V. M., Estrada-de los Santos, P., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., et al. (2004). *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *Int. J. Syst. Evol. Microbiol.* 54, 2155–2162. doi: 10.1099/ijs.0.02879-0

Rodrigues, J. L., Pellizari, V. H., Mueller, R., Baek, K., Jesus Eda, C., Paula, F. S., et al. (2013). Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 110, 988–993. doi: 10.1073/pnas.1220608110

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., et al. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* 4, 1340–1351. doi: 10.1038/ismej.2010.58

Schneider, D., Arp, G., Reimer, A., Reitner, J., and Daniel, R. (2013). Phylogenetic analysis of a microbialite-forming microbial mat from a hypersaline lake of the Kiritimati atoll, Central Pacific. *PLoS ONE* 8:e66662. doi: 10.1371/journal.pone.0066662

Shen, J. P., Zhang, L. M., Di, H. J., and He, J. Z. (2012). A review of ammonia-oxidizing bacteria and archaea in Chinese soils. *Front. Microbiol.* 3:296. doi: 10.3389/fmicb.2012.00296

Soares-Filho, B. S., Nepstad, D. C., Curran, L. M., Cerqueira, G. C., Garcia, R. A., Ramos, C. A., et al. (2006). Modelling conservation in the Amazon basin. *Nature* 440, 520–523. doi: 10.1038/nature04389

Spang, A., Poehlein, A., Offre, P., Zumbrägel, S., Haider, S., Rychlik, N., et al. (2012). The genome of the ammonia-oxidizing Candidatus Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Environ. Microbiol.* 14, 3122–3145. doi: 10.1111/j.1462-2920.2012.02893.x

Teske, A., and Sørensen, K. B. (2008). Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J.* 2, 3–18. doi: 10.1038/ismej.2007.90

Tischer, A., Potthast, K., and Hamer, U. (2014). Land-use and soil depth affect resource and microbial stoichiometry in a tropical mountain rainforest region of southern Ecuador. *Oecologia* 175, 375–393. doi: 10.1007/s00442-014-2894-x

Tripathi, B. M., Kim, M., Lai-Hoe, A., Shukor, N. A., Rahim, R. A., Go, R., et al. (2013). pH dominates variation in tropical soil archaeal diversity and community structure. *FEMS Microbiol. Ecol.* 86, 303–311. doi: 10.1111/1574-6941.12163

Tripathi, B. M., Kim, M., Singh, D., Lee-Cruz, L., Lai-Hoe, A., Ainuddin, A. N., et al. (2012). Tropical soil bacterial communities in Malaysia: pH dominates in the equatorial tropics too. *Microb. Ecol.* 64, 474–484. doi: 10.1007/s00248-012-0028-8

Tripathi, B. M., Lee-Cruz, L., Kim, M., Singh, D., Go, R., Shukor, N. A., et al. (2014). Spatial scaling effects on soil bacterial communities in Malaysian tropical forests. *Microb. Ecol.* 68, 247–258. doi: 10.1007/s00248-014-0404-7

Wang, Y., and Qian, P. Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS ONE* 4:e7401. doi: 10.1371/journal.pone.0007401

Wemheuer, B., Taube, R., Akyol, P., Wemheuer, F., and Daniel, R. (2013). Microbial diversity and biochemical potential encoded by thermal spring metagenomes derived from the Kamchatka Peninsula. *Archaea* 2013, 136714. doi: 10.1155/2013/136714

Zhou, J., Wu, L., Deng, Y., Zhi, X., Jiang, Y. H., Tu, Q., et al. (2011). Reproducibility and quantitation of amplicon sequencing-based detection. *ISME J.* 5, 1303–1313. doi: 10.1038/ismej.2011.11

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Specific microbial gene abundances and soil parameters contribute to C, N, and greenhouse gas process rates after land use change in Southern Amazonian Soils

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Ecological processes regulating soil carbon (C) and nitrogen (N) cycles are still poorly understood, especially in the world's largest agricultural frontier in Southern Amazonia. We analyzed soil parameters in samples from pristine rainforest and after land use change to pasture and crop fields, and correlated them with abundance of functional and phylogenetic marker genes (*amoA*, *nirK*, *nirS*, *norB*, *nosZ*, *nifH*, *mcrA*, *pmoA*, and 16S/18S rRNA). Additionally, we integrated these parameters using path analysis and multiple regressions. Following forest removal, concentrations of soil C and N declined, and pH and nutrient levels increased, which influenced microbial abundances and biogeochemical processes. A seasonal trend was observed, suggesting that abundances of microbial groups were restored to near native levels after the dry winter fallow. Integration of the marker gene abundances with soil parameters using path analysis and multiple regressions provided good predictions of biogeochemical processes, such as the fluxes of NO_3, N_2O, CO_2, and CH_4. In the wet season, agricultural soil showed the highest abundance of nitrifiers (*amoA*) and Archaea, however, forest soils showed the highest abundances of denitrifiers (*nirK*, *nosZ*) and high N, which correlated with increased N_2O emissions. Methanogens (*mcrA*) and methanotrophs (*pmoA*) were more abundant in forest soil, but methane flux was highest in pasture sites, which was related to soil compaction. Rather than analyzing direct correlations, the data integration using multivariate tools provided a better overview of biogeochemical processes. Overall, in the wet season, land use change from forest to agriculture reduced the abundance of different functional microbial groups related to the soil C and N cycles; integrating the gene abundance data and soil parameters provided a comprehensive overview of these interactions. Path analysis and multiple regressions addressed the need for more comprehensive approaches to improve our mechanistic understanding of biogeochemical cycles.

Keywords: tropical rainforest, biogeochemical cycles, microbial indicators, qPCR, soil processes

Introduction

Biogeochemical processes regulating the carbon (C) and nitrogen (N) cycles are still poorly understood, mainly because of the lack of adequate microbial indicators. Recent studies have shown the potential for using cultivation-independent measures, and that the quantified marker genes are good indicators of the associated biogeochemical processes in the C and N cycles (Morales et al., 2010; Petersen et al., 2012; Levy-Booth et al., 2014; Rocca et al., 2014). This approach includes the analysis of key protein-encoding genes related to these processes and their correlation and/or fit into models with standard chemical and environmental parameters (e.g., C and N forms, pH, temperature, and water content).

Several genes have been used as molecular markers to determine the abundance of a respective microbial group in soils (e.g., 16S rRNA genes; Petersen et al., 2012), and biogeochemical processes have been quantified using specific genes related to indicative enzymatic steps in the C or N cycles (Supplementary Table S1). In the N cycle, the *nifH* gene (encoding nitrogenase, that reduces N_2 from the atmosphere to ammonium) is related to N-fixation; *amoA* (ammonia monooxygenase) is related to nitrification; and *nirK* (copper nitrite reductase), *nirS* (iron nitrite reductase), *norB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase) are related to denitrification (Levy-Booth et al., 2014). In the methane cycle, the *mcrA* gene (methyl coenzyme M reductase) is linked exclusively to methanogens; and the *pmoA* gene (methane oxygenase) to methane-utilizing prokaryotes *(*methanotrophs), among several other examples (Rocca et al., 2014).

An example of the application of this approach is the study of Petersen et al. (2012), who quantified the *amoA*, *nirK*, *nirS*, and *nosZ* genes to estimate nitrification and denitrification potential in Alaskan soils, and determined them suitable indicators of these processes. Other examples are given in a review by Levy-Booth et al. (2014), in which the authors reviewed studies that had employed these and other genes as indicators of transformations within the N cycle. Recently, Rocca et al. (2014) performed a meta-analysis showing that gene quantification is still a novel approach and is constantly improving. In a comparative analysis of 59 selected studies, even though these studies were from different groups and used independent methods, the authors found significant correlations of gene quantification with C and N cycle processes ($r = 0.30$, $P < 0.001$). Based on this information, we argue that the use of gene quantification goes beyond the improvement of predictive models, and has the potential to provide information about the dynamics and ecology of the organisms directly related to these processes.

Most models attempting to predict C, N, and greenhouse gas (GHG) dynamics in soils rarely acknowledge detailed microbiological parameters (Bouwman, 1998; Rocca et al., 2014). Although the models mention the microbial processes involved, they rarely present data about the specific microbial species or functional groups which mediate these processes. Microbial information is commonly limited to general indicators such as microbial biomass, and the focus is on microbial activity alone. However, microbial activity is a consequence of the concentration of microbial enzymes and the environmental conditions that regulate their activity (e.g., substrate, temperature, available water, and O_2; Burns et al., 2013). Since soil is a complex environment for biochemical studies, the improvement of techniques for soil enzymes has been a constant challenge (Burns et al., 2013), and consequently, the physico-chemical environmental parameters which regulate enzyme activity are solely used to explain activity (e.g., "hole-in-the pipe" model; Bouwman, 1998). Methods are required that also include the abundance and ecology of the respective microorganisms that mediate the underlying process, to provide a better scientific understanding of these microorganisms and to potentially improve model accuracy (Morales et al., 2010; Petersen et al., 2012; Levy-Booth et al., 2014; Rocca et al., 2014).

Currently the best approach to identify and quantify specific microbial groups and/or enzymes related to processes of the C and N cycle is the use of cultivation independent molecular tools, e.g., the quantification of marker genes that encode the process-related enzymes (Morales et al., 2010; Petersen et al., 2012). The mRNA transcripts are not a valid parameter, since mRNA is a transitory and unstable molecule, and RNA transcripts can vary from a dozen copies to a thousand copies per cell, changing numbers within minutes depending on environmental conditions (Deutscher, 2006; Rocca et al., 2014). Accurate protein detection and quantification is still a challenge in soils (Levy-Booth et al., 2014). The quantification of genes based on genomic DNA is a powerful approach to detect the potential (dependent on other factors such as substrate availability, water and O_2) of a soil to perform the associated process (Levy-Booth et al., 2014; Rocca et al., 2014). Furthermore, the quantification of such genes enables an approximate quantification of microorganisms that belong to that functional group (the copy numbers of these genes are generally one to two copies per cell). Moreover, this strategy enables the study of the microbial organisms that perform these processes, information which is usually missing from biogeochemical approaches that focus only on environmental physico-chemical parameters, and incorporates important information about the ecology of these organisms in soils (Petersen et al., 2012; Levy-Booth et al., 2014; Rocca et al., 2014).

Here we applied the quantification of specific functional marker genes to provide important insights into consequences of land use change from pristine rainforest to agricultural uses in Southern Amazonia, the largest agriculture frontier worldwide. In Brazil, agriculture is of great social, economic, and environmental importance. Because of the growing domestic and global demand for agricultural products, the country has continually expanded its agricultural activities in the past decades. Today Brazil is the second biggest soybean and livestock producer in the world (Food Agriculture Organization [FAO], 2014), and these agricultural areas are constantly expanding from their origins in the south further north in the direction of the Amazonian rainforest. Within Brazil the state of Mato Grosso in Southern Amazonia ranks as the biggest producer of soybeans, and contains the largest area covered by pasture in Brazil. This region represents the most dynamic agricultural expansion worldwide, where tropical rainforests have been converted into

pasture and crop lands, but little is known about the impacts of these land use changes on soil biogeochemical cycles (Galford et al., 2010, 2011).

Following deforestation, not only plant biomass but also the stocks of total C and N in soil can become mobilized and be partially emitted in the form of the greenhouse gasses CO_2, CH_4, and N_2O (Cerri et al., 2006). Some studies conducted in Southern Amazonia have evaluated the impact of land use change on soil organic matter (SOM) and GHG fluxes (Neill et al., 1997; Carvalho et al., 2010; Maia et al., 2010, Galford et al., 2011), and found that losses of soil C and N stocks are greatly modified by agricultural management. Conventional tillage, overgrazing, and low chemical fertility can lead to a significant loss of soil C, but specific agricultural conservation practices, such as no-tillage, can increase SOM accumulation in these tropical soils (Carvalho et al., 2010; Maia et al., 2010). Although C, N, and GHG cycles have been studied in parts of Southern Amazonia, little is known about the microorganisms responsible for these processes in this region (Carvalho et al., 2010).

The objective of this study was to analyze *in situ* how the abundance of microbial functional groups related to the soil C and N cycles shifted in response to land use change from rainforest to agricultural use in Southern Amazonia. Furthermore, we investigated whether these changes were correlated with changes in soil C and N content, or with soil GHG fluxes (CO_2, CH_4, and N_2O). We compared a pristine rainforest with adjacent pasture and soybean sites established on the same soil approximately 25 years ago following deforestation. For further comparison, we included another agricultural site within the same ecotype, which has soybeans established only 2 years after forest conversion, enabling the detection of short-term effects on biogeochemical cycles in this region.

The primary hypothesis for this investigation was that the effects of land use change on the abundance of microbial functional groups are related to the total soil C and N concentrations, and to GHG fluxes in Southern Amazonia. A secondary hypothesis states that the abundances of microbial marker genes are appropriate indicators to model processes within the respective biogeochemical cycles.

Materials and Methods

Site Survey and Soil Sampling

This study was conducted on three farms nearby the municipality of Sinop, Mato Grosso State, Brazil, one of the most important agricultural regions in Southern Amazonia (Galford et al., 2010). Typical farms with similar edapho-climatic conditions and known land use history were chosen (Supplementary Table S2). The forest on the chosen farms was identified as a semideciduous mesophytic forest, with Amazonian species affinity (Ackerly et al., 1989), and the soils were classified as Red Oxisol with clay texture. In the past, the same forest type was removed at a large scale from adjacent areas (same soil type) and converted to agriculture. The climate is megathermic, with wet summers and short, dry winters, or 'Am' by the Köppen-Geiser climate classification. The average temperature is 24.1°C, and the average annual precipitation is 2171 mm (Vourlitis et al., 2008). Old established soybean fields in this region are typically cultivated using a no-till system with double cropping with corn or sorghum, while new soybean fields after deforestation are typically cultivated using conventional tillage (Carvalho et al., 2007). Both soybean fields receive annual fertilization, periodic liming (Ca and Mg carbonates), and pesticides; in contrast, pastures rarely receive annual fertilization or liming (Lammel et al., 2015). The land use types sampled and their geographic coordinates were: 12°05′22″S, 55°28′24″W (25 years soybean field), 12°05′26″S, 55°28′35″W (pasture), 12°05′34″S, 55°28′43″W (adjacent forest), and 11°44′49″S 56°15′14″W (2 year soybean field and adjacent forest; Supplementary Table S2).

Soil samples were surveyed during two different time periods. The first sampling was in the 1st week of November 2010, at the beginning of the wet season, and just prior to soybean seeding. Samples were taken a second time in January 2011, in the middle of the wet period, and immediately after soybean flowering, termed stage R3 (Fehr et al., 1971). The characteristic dry period of Southern Amazonia falls between June and September, therefore sampling efforts were focused on the beginning (November) and the intermediate wet season (January), assuming higher microbial activity during this time because of higher soil moisture, and higher average temperature. This period also coincides with the soybean growth stages of seedling (October–November) and maximum growth (January). For the two soil surveys DNA was extracted and gene quantification performed.

Soil was sampled from two (November 2010) or six replicated plots (January 2011) for each land use type. Within each replicated plot five soil samples were surveyed, and a total of 10 and 30 samples were studied per land use type at each sampling time, respectively. Replicated plots were located from dozens to 100 m away from each other and were characterized by the same topography and soil type (Red Oxisol). This is the predominant soil type in this agricultural region, and to reduce potential bias caused by soil variation we only surveyed samples of this soil type (Maia et al., 2010). Land use history and soil profile analyses confirmed that the agricultural sites had been converted from the same adjacent forest and soil types. In each of the replicated plots, soil samples were collected in a cross shaped design with four samples in the cardinal directions located 20 m equidistant to the fifth sample in a central position.

The soil survey in the middle of the wet season (January 2011) was performed together with a soil gas flux survey, with the aim of correlating soil and microbial parameters with the quantification of GHG fluxes. For this, we collected soils from six replicated plots for each land use type within a 1 week period (January 15–19), as described above. Within that week, two replicate sites were surveyed for each land use type in three subsequent sampling events spaced 2 days apart, January 15, 17, and 19. Thus, a total of 30 samples were analyzed for each land use type, originating from six replicated plots, each with five sub-samples. At the same time two replicated plots were sampled from the 2 year-old soybean sites, yielding an additional 10 samples (surveyed on January 16).

After removing the litter layer, soil was sampled from 0 to 17 cm using sterile PVC tubes. Soil cores were immediately

packaged in sampling bags and placed on ice, to be frozen later on the day of sampling at −20°C (Lammel et al., 2015). One week later, the frozen soil cores were used to extract DNA, nitrate, and ammonium. All samples were individually processed to avoid cross contamination, and all equipment was disinfected with 80% ethanol prior to sampling. In the survey during the wet season, soil cores were collected immediately after GHG sampling inside the ring base of the gas sampling chamber to ensure that the soil cores corresponded to their respective *in situ* gas fluxes (for details on gas sampling see below).

Chemical Analysis

Soil samples were processed according to the standard methods for Brazilian tropical soils used by the Agronomic Institute of Campinas (IAC; Cantarella et al., 1998). The pH values were measured in 0.01 M $CaCl_2$; exchangeable cations (K^+, Ca^{2+}, and Mg^{2+}) and available P were extracted using ion exchange resins; the trace elements Cu, Fe, Mn, and Zn were extracted by diethylenetriaminepentaacetic acid and triethanolamine with a pH of 7.3, and B was thermally extracted in water (Cantarella et al., 1998; Carvalho et al., 2007). Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted by adding 4 g frozen soil to 40 ml of 1 M KCl; the suspension was agitated for one h, and filtered through quantitative filter paper at a pore size of 8 μm (Lammel et al., 2015). The extract was analyzed in a flow injection analysis system. NH_4^+ concentration was determined by conductivity detection, and NO_3^- was reduced in a Cd column, and measured colorimetrically (Cerri et al., 2006). Total C and total N were determined using a C/N Analyzer CN-2000 (LECO, St. Joseph, MI, USA; Carvalho et al., 2010).

Quantitative PCR Analysis

Total DNA was extracted from 0.25 g of soil using the Power Lyzer Power Soil DNA Isolation Kit (MoBio, Solana Beach, CA, USA), according to the manufacturer's instructions, and stored at −20°C. For each of the two (November) or six (January) replicated plots, DNA extraction was performed independently for each of the five sub-samples (soil cores). The resulting five DNA extracts were pooled at equal volumes, resulting in one DNA sample for each replicated plot ($n = 2$ or 6), and used as templates for qPCR.

Quantitative PCR was performed in an Opticon2 device (Bio-Rad, Berkeley, CA, USA) in 96-well plates. All analyses were performed at least in duplicate. Each reaction was composed of 1 μl of extracted template DNA, 0.2 μl of KlenTaq DNA polymerase, 1× KlenTaq buffer (DNA Technology, St. Louis, MO, USA), 0.5 μg of T4 Gene 32 Protein, to both increase template detection sensitivity and suppress humic acid inhibition (Tebbe and Vahjen, 1993; Kermekchiev et al., 2009), 1× EvaGreen (Biotium, Hayward, CA, USA), 0.25 mM of dNTPs, primer pairs as described (Supplementary Table S1), and molecular grade water to a final volume of 20 μl (Rotthauwe et al., 1997; Hallin and Lindgren, 1999; van Elsas et al., 2000; Throbäck et al., 2004; Rösch and Bothe, 2005; Yu et al., 2005; Henry and Bru, 2006; Dandie et al., 2007; Mcdonald et al., 2008; Steinberg and Regan, 2008; Claesson et al., 2010). Standards for quantification were prepared from PCR amplified genes from environmental DNA using each primer set, and dilutions were employed as qPCR standards using the method described by Hou et al. (2010). The thermocycler was programmed with an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C denaturation for 15 s, annealing (temperature specified in **Table 1**) for 30 s, and an extension at 68°C for 30 s, followed by a final extension cycle of 5 min at 68°C. All primer pairs were chosen according to the literature available in 2010, and amplification protocols were optimized for our laboratory conditions (for details, refer to **Table 1**). Melting curve analyses were performed from 68 to 95°C, and standard curves had R^2 higher than 0.99. The amplified fragments were also confirmed in agarose gel for specificity and size (Petersen et al., 2012). No significant inhibition was observed when standards were compared to soil extracts spiked with the addition of standards. Amplification efficiencies were 84 ± 9%, a value that is in the range of similar studies (Meyer et al., 2013; Jones et al., 2014).

GHG Analysis

Soil gas fluxes were measured using a two-piece static polyvinyl chloride plastic chamber (Steudler et al., 1989). Five chamber-bases, separated from each other by 20 m, were inserted 2 cm into the soil at each sampling site in a cross design ($n = 30$, consisting of five chambers for each of the six replicated plots). After mounting the gas-tight chamber lid, headspace gas samples of 10 ml were surveyed using nylon syringes at the beginning of each incubation (time 0), and 10 and 20 min thereafter. Individual samples were transferred to glass tubes sealed with air tight stoppers and crimped for storage until analysis. Air chamber temperature, soil temperature at 5 and 10 cm depths, and atmospheric barometric pressure were measured. All gas samples were analyzed immediately after the survey, within a week, using the gas chromatograph GC-2014-GHG (Shimadzu, Kyoto, Japan). The fluxes of each gas were determined by calculating the changing concentration in the chambers as a function of incubation time, and adjusted for pressure, chamber volume, and temperature (Cerri et al., 2006).

Statistical Analysis

The data were analyzed as a mixed model considering a nested design, where the replicated sites were nested with the surveyed days ($n = 6$ for qPCR data) and individual soil cores ($n = 30$ for all forms of C and N, and GHG fluxes; Pinheiro and Bates, 2000). Data were Box–Cox transformed and submitted to an ANOVA, and when a significant effect was observed, a Tukey *post hoc* test was performed ($P < 0.05$). All tables and graphs are shown as averages of the replicate plots ($n = 2$ for the November 2010 samples, $n = 6$ for the January 2011 samples, with the exception of soybean 2 years where $n = 2$). Pearson correlation and principal components analysis (PCA) were performed to test relationships between the variables, and n-MANOVA to check if the land use types were statistically different (Anderson, 2001). All analyses were run using the software R, and the libraries car, lme, and vegan (R Core Team, 2013).

To further evaluate the fit of the marker gene abundances with environmental parameters in biogeochemical processes, multiple regression, and path analysis were performed. Five models were

tested, considering C degradation and CO_2 flux, nitrification, denitrification, and methane flux (Supplementary Figure S1). Path analyses were performed as described by Petersen et al. (2012), based on the reduction of the full models, and selection of the best models where all paths were statistically significant. Some of our data presented a moderately skewed distribution, thus path models were analyzed using Bollen–Stine bootstraps in the R-library Lavaan (Rosseel, 2012). The general fit of the model is shown, with a desirable $P > 0.05$, Comparative Fit Index (CFI) > 0.93 (0 low fit, 1 best fit), and a Standardized Root Mean Square Residual (SRMR) < 0.08, with the individual paths having $P < 0.05$ (Rosseel, 2012). Multiple regressions were performed for both parametric models and general additive models using smoothing spline within the R-library mgcv, which supports semi and non-parametric distributions (Wood, 2006).

Results

Land use change from forest to pasture or soybean resulted in several changes in soil physicochemical and microbial parameters. Forest soil was characterized by higher concentrations of total C and N as well as higher available mineral N values (NH_4^+ and NO_3^-) compared to all agricultural sites (**Table 1**). After conversion to agriculture, pH values and soil nutrient concentrations increased (P, Ca, Mg), most notably in the soybean fields (**Table 1** and Supplementary Table S3). These chemical changes paralleled shifts in abundances and activities of microbial functional groups (**Table 1**).

The initial characterization of gene abundance in soils was made in November, at the beginning of the wet season. No statistically significant differences in gene abundance were observed among land uses in November (Tukey, $P < 0.05$; Supplementary Figure S2), suggesting that the effect of land use change on the microbial community was low. Significant differences in gene abundances were obtained between November and January samples ($P < 0.05$). In January, during the middle of the wet season, the differences between forest and derived sites were evident (**Table 1**, $P < 0.05$), therefore the present study focused on this period. All the results below refer to the January samples (to avoid confusion, results from the November samples are given only in the Supplementary Information).

TABLE 1 | Selected soil attributes, and gene abundances and gas fluxes in the surveyed areas in the wet season (see the full data set in Supplementary Table S3).

Attribute	Unit	Forest	Soybean 2y	Soybean 25y	Pasture
Soil Chemistry					
C	$mg.g^{-1}$	2.9a[1]	2.6b	2.3c	2.5
N	$mg.g^{-1}$	0.17a	0.14c	0.14c	0.15b
NH_4	$\mu g.g^{-1}$	4.7a	0.7c	2.2b	2.6b
NO_3	$\mu g.g^{-1}$	3.0a	0.8b	0.7b	0.9b
pH	–	3.9c	5.1a	5.0a	4.6b
Ca	$mmol_c.dm^{-3}$	2.0c	25.0a	27.7a	11.7b
Mg	$mmol_c.dm^{-3}$	2.0c	19.7a	6.3b	5.7b
P	$mg.dm^{-3}$	4.3b	16.0a	13.7a	3.3b
Cu	$mg.dm^{-3}$	0.2b	0.7a	0.4ab	0.1b
Marker genes					
16S Archaea	copies.g $soil^{-1}$	8.8E + 06b	7.4E + 06c	1.3E + 07a	9.1E + 06b
16S Bacteria	copies.g $soil^{-1}$	2.7E + 09a	2.1E + 08c	3.5E + 08c	1.1E + 09b
18S Fungi	copies.g $soil^{-1}$	6.0E + 06a	1.6E + 05b	1.9E + 05b	4.9E + 06a
N-cycle genes					
nifH	copies.g $soil^{-1}$	2.1E + 07a	2.9E + 06c	5.1E + 06c	1.5E + 07b
amoA Archaea	copies.g $soil^{-1}$	5.8E + 05d	1.5E + 06c	6.0E + 06a	4.0E + 06b
amoA Bacteria	copies.g $soil^{-1}$	4.8E + 05a	2.0E + 05b	1.7E + 05b	2.3E + 05b
norB	copies.g $soil^{-1}$	2.0E + 06a	5.7E + 05b	1.5E + 06a	2.4E + 06a
nirK	copies.g $soil^{-1}$	7.4E + 06a	5.9E + 05c	2.0E + 06c	4.0E + 06b
nirS	copies.g $soil^{-1}$	2.2E + 06b	4.5E + 05c	7.6E + 05c	4.6E + 06a
nosZ	copies.g $soil^{-1}$	2.3E + 07a	1.7E + 06c	1.9E + 06c	8.8E + 06b
Methane genes					
mcrA	copies.g $soil^{-1}$	2.1E + 05a	1.4E + 04c	2.3E + 04c	7.2E + 04b
pmoA	copies.g $soil^{-1}$	6.7E + 07a	9.8E + 06c	1.0E + 07c	3.1E + 07b
Gasses					
CO_2 Flux	mg $C\text{-}CO_2.m^2$ soil. h^{-1}	129b	258a	98b	111b
CH_4 Flux	μg $C\text{-}CH_4.m^2$ soil. h^{-1}	−7b	−14b	8b	46a
N_2O Flux	μg $N\text{-}N_2O.m^2$ soil. h^{-1}	33a	9b	4b	4b

[1] *Values with the same letter in one row are not different by Tukey's post hoc test ($p < 0.05$).*

Microbial Abundance and Activity

Different land uses were characterized by distinct patterns of microbial abundance. Abundances of Bacteria and Fungi were highest in forest sites, while Archaea dominated old soybean fields (**Table 1**). Bacterial and fungal abundance were negatively correlated with pH, soil bases (Ca, Mg, K), P, and Zn, and positively correlated with total N, NO_3^-, CEC, H^++Al^{+3}, and Fe. In addition, Bacterial abundance was also positively correlated with total C, CEC, B, Fe, Mn, and B (Supplementary Table S4). Microbial activity, represented by CO_2 flux, was more intense in the new soybean soils, and no difference was observed among the other land use types (**Table 1**).

To further investigate the interaction among the variables, two techniques were used to test models with these variables and to explain the soil respiration. The first was path analysis, which tested selected combinations of the variables based on an initial full model (Supplementary Figure S1). That initial full model was reduced to a significant model where all the paths were significant (**Figure 1A**). In this path diagram, the soil C negatively influenced Archaeal abundance, the soil pH negatively influenced Bacterial abundance, and all of them in addition to OM influenced the CO_2 flux. The numbers on the top of each variable boxes represent unexplained variation ($1 - R^2$), which represents the effect of unmeasured variables and measurement error (Petersen et al., 2012). This means that in this model only 0.23 of the CO_2 Flux was not explained by this model; and that Archaea and Bacteria contributed a little to the model, with 0.83 and 0.63 of their variance not explained by this model. The other technique used was stepwise multiple regressions, and in this case, there was no guided dependency among variables as that stated in the path analysis (Supplementary Information "Regressions"). Full models were than tested and reduced for models where all the coefficients were statically significant and the best Akaike information criterion (AIC) achieved. The coefficients of the best analysis were then used in a linear regression against the CO_2 flux, and achieved an R^2 of 0.97 and $P < 0.001$ (**Figure 2A**). Both independent techniques showed that the abundance of 16S genes of Archaea and Bacteria contributed to the explanation of the CO_2 fluxes from the analyzed samples.

N-Cycle Dynamics

The abundance of N-fixers, based on *nifH* genes, was highest in forest soils, followed by pasture soils. Archaeal ammonium oxidizers (AOAs) were numerically dominant at all sites, and overall abundance (*amoA* Archaea) was highest in the old soybean fields, and lowest in forest soils. Bacterial ammonium oxidizers (AOB) were more abundant in the forest sites (**Table 1**). The abundance of denitrification genes was highest in forest soils, intermediate in pasture, and lowest in soybean fields (**Table 1**). Significant correlations and relationships between related genes (*nirK* and *nosZ*) were found, and also between them and N, NO_3^-, NH_4^+, and N_2O (Supplementary Table S4). Interestingly, abundances of *nifH*, *amoA* Bacteria, *nirK,* and *nosZ* genes were correlated between each other (in fact, they are all present in some bacteria, such as *Rhizobium* sp.). Interactions among the variables were further characterized by 3D plots and multivariate techniques (**Figures 3** and **4**).

To explain NH_4^+ concentrations in soil, a 3D bubble plot showed the relationships among soil N, N-fixers (*nifH*) and litter N (litter data from Lammel et al., 2015) (**Figure 3A**, a linear-mixed model for this data showed $R^2 = 0.61$, AIC = 74). These relationships were also demonstrated by a path analysis

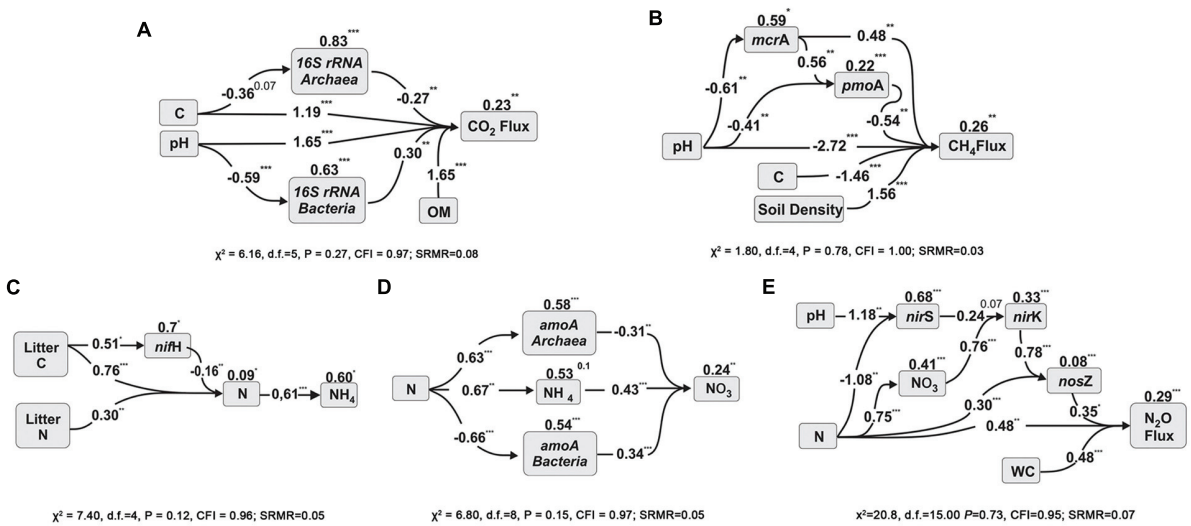

FIGURE 1 | Path analysis diagrams for microbial gene abundances and CO_2 fluxes (A); for methane dynamics (B); and for N-cycle related processes, NH_4^+ prediction (C), NO_3^- prediction (D) and N_2O fluxes (E). The diagrams show the relationships among the selected variables and the influence of soil chemical factors in soils across land use types in Southern Amazonia in the middle of the wet season (for more information please see the Methods section). The numbers listed within arrows are standardized path coefficients (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, or x $P <$ x). The numbers on the top of the variable boxes represent unexplained variation ($1 - R^2$) which represents the effect of unmeasured variables and measurement error. The litter C and N data are from Lammel et al. (2015).

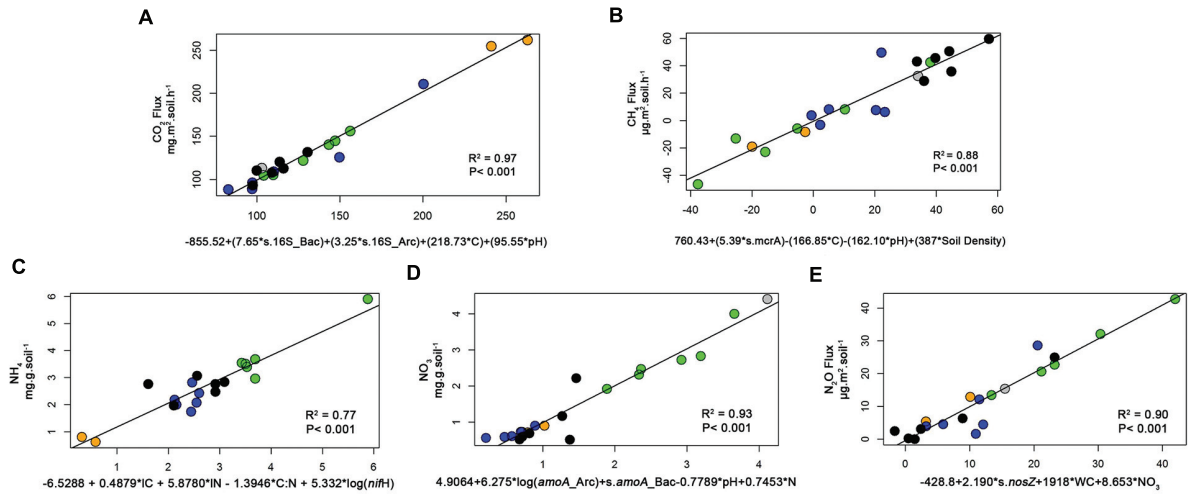

FIGURE 2 | Linear regression analyses (reporting the R^2 and P-value) between selected processes, CO_2 fluxes (A), CH_4 fluxes (B), NH_4^+ prediction (C), NO_3^- prediction (D), N_2O fluxes (E), and an equation made with the coefficients from the best multiple regression result for each process (for details please see Methods and the Supplementary Information "Regressions"). These graphs demonstrate the fit (R^2) of our data in the prediction equations based on the multiple regression analyses. Data are shown for four different land use types, forest (green), pasture (black), soybean 25 years (blue), soybean 2 years (orange squares, and adjacent forest in gray).

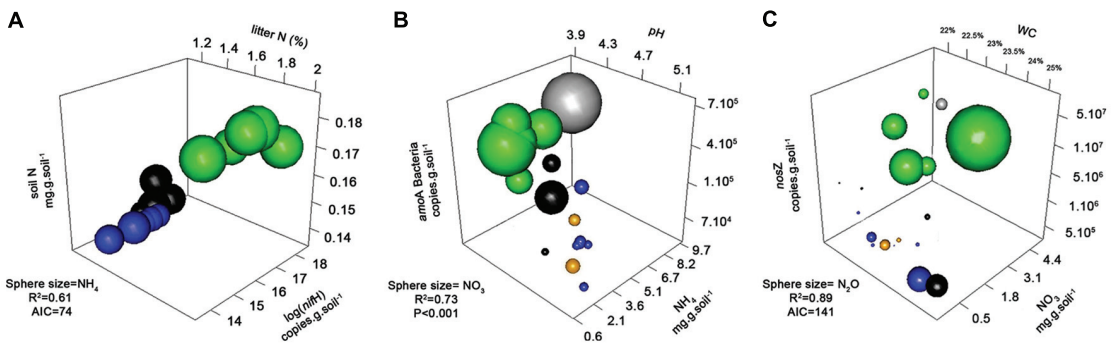

FIGURE 3 | 3-D bubble plots based on univariate data to explain the products NH4+ (A), NO_3^- (B), and N_2O Flux (C). The axes are the predictor variables and the sphere sizes the respective products. For each graph, its significance was tested by a regression analysis, reporting its R^2 and significance (P or AIC). Data are shown for four different land use types, forest (green), pasture (black), soybean 25 years (blue), soybean 2 years (orange squares, and adjacent forest in gray).

(**Figure 1C**). The path diagram indicated that litter C, litter N, N-fixers, and soil N were statically significant to explain NH_4^+ concentrations, however, the NH_4^+ unexplained variation ($1 - R^2$) in this model was 0.60. Soil N had the highest fit to the path diagram, and had only 0.09 of unexplained variation. The best multiple regression result (Supplementary Information "Regressions") incorporated soil C:N, litter C, litter N, and *nifH* gene as variables; when their coefficients were used in a linear regression with NH_4^+, the R^2 was 0.77 and $P < 0.001$ (**Figure 3C**).

To explain NO_3^- in soil, a 3D bubble plot showed the relationships among soil NH_4^+, nitrifiers (*amoA* Bacteria) and pH (**Figure 3B**, a linear model for this data showed $R^2 = 0.73$, $P < 0.01$). The path diagram indicated that soil N, NH_4^+, *amoA* Archaea and *amoA* Bacteria were statically significant to explain the NO_3^- concentrations, and it presented 0.23 of unexplained variation in this model (**Figure 1D**). Soil NH_4^+ had the best fit to this path diagram, and had 0.53 of unexplained variation. The best multiple regression result (Supplementary Information "Regressions") had the genes *amoA* Archaea and Bacteria, pH and soil N as variables; when their coefficients were used in a linear regression with NO_3^-, the R^2 was 0.93 and $P < 0.001$ (**Figure 2D**).

To explain N_2O flux, a 3D bubble plot showed the relationships among water content (WC), NO_3^- and *nosZ* gene abundance (**Figure 3C**, a linear-mixed model indicated $R^2 = 0.89$, AIC = 141). The path diagram indicated that soil N, pH, NO_3^-, *nirS* and *nirK* and *nosZ* genes and WC were statically significant to explain N_2O flux, and it presented 0.29 of unexplained variation in this model (**Figure 1E**). The *nosZ* gene abundance had the best fit to the path diagram, and had only 0.08 of unexplained variation. The best multiple regression result

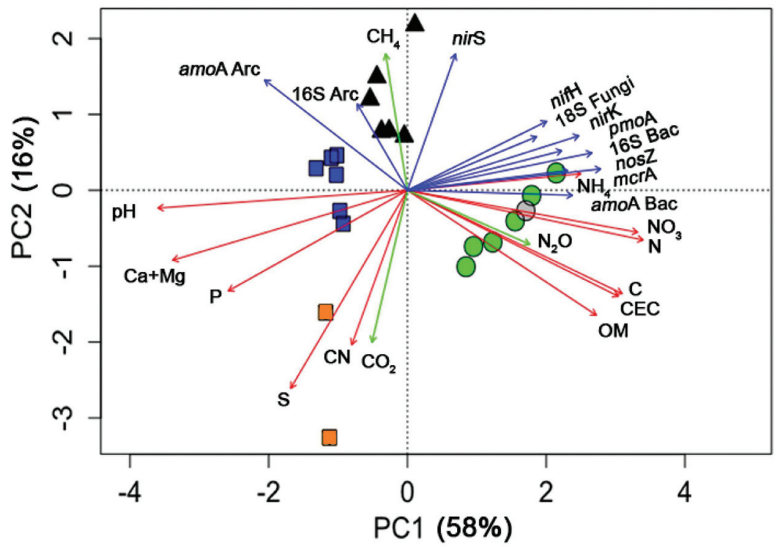

FIGURE 4 | Principal Components Analysis of land uses (symbols) and soil parameters (vectors) in Southern Amazonia in the middle of the wet season. Data are shown for four different land use types, forest (●), pasture (▲), soybean 25 years (■), soybean 2 years (■, and adjacent forest in a gray circle); and vectors for the soil chemical parameters, microbial genes, and GHG fluxes.

(Supplementary Information "Regressions") had *nosZ*, WC and NO_3^- as variables; when their coefficients were used in a linear regression with N_2O, the R^2 was 0.90 and $P < 0.001$ (**Figure 2E**).

Methane Dynamics

Methane flux in forest soils and soybean fields varied from consumption to production, and was characterized by high spatial variation (**Table 1**; **Figure 2B**). On average, new soybean sites acted as a methane sink, while forest and old soybean soils had close to a neutral flux; pasture was clearly a methane source.

Abundances of methanogenic (*mcrA*) and methanotrophic (*pmo*A) genes were similar for each land use and were strongly correlated (0.82, $p < 0.001$); gene abundances were highest in forest sites, and lowest in soybean soils (**Table 1**). No direct correlations between abundances of these genes and methane fluxes were detected (Supplementary Table S4). Path diagram analysis indicated that soil pH, C, bulk density and the abundance of *mcrA* and *pmoA* genes were statically significant to explain CH_4, and it presented 0.26 of unexplained variation in this model (**Figure 3D**). The best multiple regression result had soil C, pH, density and the *mcrA* gene as variables (Supplementary Information "Regressions"); when their coefficients were used in a linear regression with CH_4, the R^2 was 0.88 and $P < 0.001$ (**Figure 2B**).

Linking Gene Abundances, Soil Parameters, and Processes

Gene abundances were correlated with soil chemistry and total C and N dynamics. The strongest correlations were obtained between genes and pedological factors involved in denitrification, namely NO_3^-, *nirK* and *nosZ* gene abundances, and N_2O flux, but several other weaker correlations were also observed (Supplementary Table S4). Using path analysis the dependencies of these variables was tested in five models, namely microbial activity, ammonium mineralization, nitrification, denitrification, and methane dynamics (Supplementary Figure S1), and reduced models that present significant paths are shown (**Figure 1**). We also demonstrated the fit of these variables using multiple regressions, indicating that the marker genes fit well into the tested biogeochemical models (**Figure 2** and Supplementary Information "Regressions").

For a better overview of the analyzed soil parameters (genes and chemistry) and their relationships by land use type, a PCA was performed (**Figure 4**). Each variable (soil parameter) is represented by a vector, and the length of each vector indicates the strength of its contribution. The relative importance of each variable can be estimated from the perpendicular projection of each sample to its respective vector. For example, C was highest in the forest samples (if one takes a perpendicular imaginary line to the C vector, one can see that forest samples are at the top of this vector). The two main axes (PC1 and PC2) indicate the total variance of the data explained in the PCA (58 and 16%, respectively). Thus, the forest soils were best characterized by high quantities of OM, CEC, C, N_2O, N, NO_3^-, NH_4^+, and most of the marker genes. All soybean soils were characterized by high pH and soil fertility (P, Ca+Mg, and S), while soybean 2y had high soil CN and CO_2 flux and the soybean 25y and pasture soils had high abundance of *amoA* Archaea and 16S Archaea. Pasture sites were characterized by high *nirS* abundance and CH_4 flux. In addition to the PCA, n-MANOVA analysis was performed and showed that the discrimination of the land use types (as observed in the PCA by the different cluster of symbols) was statistically significant ($P < 0.05$).

Finally, to facilitate the visualization of all the presented data (gene abundances and their chemical substrates and products) in the context of C and N cycles in soils, we propose an integrated

diagram of soil chemical parameters, gene abundances, and gas fluxes for the effects of this land use change (**Figure 5**). Overall, land use affected the microbial groups, and gene abundance was a sensitive indicator that correlated positively with C and N forms and fit well into the tested biogeochemical models (**Figures 1–3**).

Discussion

In this study, we showed that the effects of land use change on microbial functional groups, accessed by their marker genes, provided useful information to explain the reported variations in total N and GHG fluxes (for CO_2, CH_4, and N_2O) in Southern Amazonia soils. While land use type, seasonal management, and soil chemistry affected microbial abundance, the abundance of specific marker genes for microbial functional groups linked to soil parameters using multivariate tools contributed to explained values of C- and N-related process rates (**Figures 1–3**).

Microbial Abundance and Activity

The highest microbial abundances, obtained in forest sites, were correlated with the highest concentrations of C, N, NH_4^+, and NO_3^- in the forest sites. These characteristics are often observed in pristine soils when compared with agricultural soils, and are explained by litter degradation and tight nutrient cycling (Brando et al., 2008; Wieder et al., 2013). A previous study indicated that these forest sites had a more extensive litter layer and a more distinct chemical composition compared to the agricultural sites (Lammel et al., 2015). Litter quantity and quality were different between forest and agricultural sites, with the natural vegetation presenting higher proportions of recalcitrant compounds and a higher C:N ratio, which supports higher C accumulation and constant N mineralization in forest soils compared with agricultural soils (Wieder et al., 2013; Lammel et al., 2015). Also, abundance of N-fixing microorganisms was highest in forest soils, which over a long time period can account for higher soil N levels (Morales et al., 2010).

Thus, we associate the highest microbial abundance in the forest sites during the wet season with the highest levels of soil C and N (**Figure 3**; Yao et al., 2000; Stevenson et al., 2014). Additionally, several aspects of agricultural management might alter the abundance of Bacteria and Fungi in soils, such as the chemical changes introduced by fertilization and pesticide application (Yao et al., 2000; Imfeld and Vuilleumier, 2012). A previous study in these areas reported agricultural management (plant cover, fertilization, and pesticides) as drivers for soil Bacterial community structures (Lammel et al., 2015). To manage plant diseases and control harmful insects and weeds during soybean growth, fungicides, insecticides, and herbicides are applied (Supplementary Table S2), which can all function as factors related to soil microbial suppression (Imfeld and Vuilleumier, 2012; Lammel et al., 2015). In our study, Archaea showed the highest abundance in old soybean soils, which is an intriguing result, as it would be expected that Bacteria would have higher abundance compared to Archaea in response to liming and fertilization (Bengtson et al., 2012; Tripathi et al., 2013). Since the opposite result was observed in the present study, we suggest that while pesticides likely suppressed Bacterial and Fungal abundance in soybean fields, other factors selected for Archaeal abundance. Archaeal abundance is reportedly variable

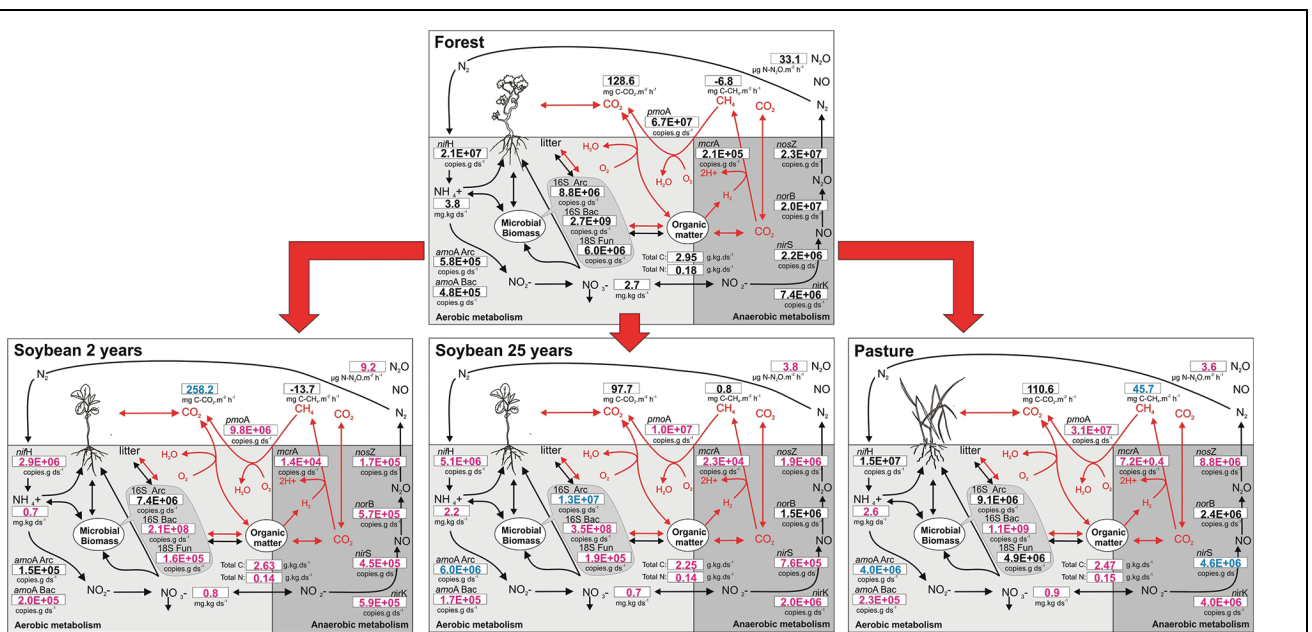

FIGURE 5 | Integrated scheme for the carbon and nitrogen cycles in response to land use change in the soils of Southern Amazonia. The abundance of soil microbial genes involved in biogeochemical cycles of C and N are shown together with their respective end products. The forest was set as the reference value representing an unchanged control. Values that are modified with land use change from forest to soybean fields or pasture are indicated in color (increases in blue, and decreases in magenta; Tukey's *post hoc* test, $P < 0.05$).

in soils at pH values below 4.7 (such as our forest sites); the higher pH value in the soybean site, the lower C:N ratio, and increased soil compaction leading to anaerobic microsites, as observed after the conversion from forest to agriculture in our study, might be drivers for the abundance of this group (Bates et al., 2011; Bengtson et al., 2012; Karlsson et al., 2012; Šibanca et al., 2014). Plant cover might be important to Archaeal abundance, since root exudates can influence the abundance of this group in the rhizosphere and in the bulk soil. Differences in tillage practices might also have an impact on Archaeal abundance; new soybean 2y had low abundance of Archaea, perhaps because conventional till practices affect the O_2 status of the soil, whereas the 25y established soybean plots under no-till might have selected for this group (Karlsson et al., 2012; Sugiyama and Yazaki, 2012). We are not able to explain Archaeal abundances with precision at this time and future studies should clarify this phenomenon.

Elevated CO_2 fluxes indicated higher microbial activity in new soybean soils. These fields were managed using conventional tillage, which is used in the 1st years after deforestation to aid in the incorporation of liming and in the decomposition of forest roots. Microbial respiration is usually more intense under these conditions in response to elevated soil pH, oxygenation by plowing, and increased levels of bioavailable carbon from the forest roots and debris (Carvalho et al., 2007; Pes et al., 2011). The opposite is observed in no-till systems, such as the old soybean and pasture fields, where native vegetation and agricultural soils produced similar CO_2 fluxes, as observed in the present study for all other land uses (Carvalho et al., 2010). These results show that not only direct correlations between genes and process rates should be investigated, but also the relationships between them and other environmental parameters. In this case both the path analysis and the multiple regressions detected the importance of C, pH and the abundance of Archaea and Bacteria in explaining the CO_2 flux rates (**Figures 1** and **2**). However, soil management factors such as oxygenation by plowing (parameter not measured) should also be taken into consideration in explaining the observed results (Carvalho et al., 2007; Pes et al., 2011).

N-Cycle Dynamics

Archaeal ammonium oxidizers were more abundant than AOB in all areas, and were most abundant in agricultural soils (Leininger et al., 2006; Meyer et al., 2013). Concurrently, AOA, similar to total Archaea, and opposing AOB, were most abundant in higher pH old soybean fields, and had lower abundance in the acidic forest soils. Since generalizations around the effect of pH are not universally applicable and should be taken with caution, we suggest that agricultural land use selected for higher abundance of AOA, as suggested by some studies (Prosser and Nicol, 2012; Jiang et al., 2014). Additionally, higher C (correlation C/AOB $R = 0.55$, $P < 0.001$) and NH_4^+ concentrations (correlation NH_4^+/AOB $R = 0.59$, $P < 0.01$) in forest sites might have selected for higher abundances of AOB in those soils than in the agricultural soils, since AOB are generally more sensitive to such drivers (Verhamme et al., 2011; Stempfhuber et al., 2014). Interestingly, AOA followed the Archaeal abundance and we are not able to explain in details at this time why old soybean fields selected for this group.

Although AOA were more abundant in all soils, AOB presented the best fit explaining NO_3^- (**Figure 3B**), similar to a previous report for potential nitrification rates in a laboratory analysis (Petersen et al., 2012). However, it is difficult to quantify nitrification rates in the field as these rates depend on multiple factors, including N availability, AOA and AOB interactions, the plant influence on the microbial community, plant uptake of NH_4^+/NO_3^-, and other environmental factors. Xia et al. (2013) was recently able to identify a better correlation of nitrification with AOB by using stable isotopes in soils cultivated with soybeans. Future studies with stable isotope probing might better delineate the contributions of AOA and AOB to soil nitrification in tropical soils under land use change.

In contrast to the distribution of nitrifiers, denitrifiers (*nirK* and *nirS* genes) were more abundant in forest and pasture sites. Abundance of *nirK*- and *nirS*-type denitrifiers differed significantly between land uses. While *nirK*-type denitrifiers were distinctly dominant in forest sites, the ratio of *nirK*/*nirS* was high in forest and soybean soils. Variable abundance ratios of *nirK* and *nirS* genes have been reported, with a trend of *nirK*/*nirS* ratios usually >1 (Bárta et al., 2010; Enwall et al., 2010; Meyer et al., 2013; Jones et al., 2014). Little is known about the drivers for the differentiation in abundance of *nirK* or *nirS*-gene denitrifiers in soils, but elevated soil pH and Cu concentration have been reported as important factors having a positive influence on *nirK* abundance (Enwall et al., 2010; Bru et al., 2011). Neither of these characteristics was observed in our data. New soybean field soils contained the highest Cu content, however, *nirK* genes were most abundant in forest soil. Plant cover and soil chemistry might be similarly important in determining the distribution of denitrifiers in soil, i.e., specific plants might select for a higher abundance of *nirK*-gene bearing denitrifiers (Bremer et al., 2007; Meyer et al., 2013).

A strong correlation was observed between abundances of *nirK* and *nosZ* genes (0.92, $p < 0.001$). However, there was no correlation between the subtraction of *nosZ* from *nirK*/*nirS* copy numbers with N_2O fluxes, as suggested by Morales et al. (2010). In fact, some bacteria that possess *nosZ* clade I (our primer target) also have the *nirK* or *nirS* genes (~67% of the analyzed genomes by Jones et al., 2008). The recently discovered *nosZ* clade II (not covered by our primers) has also been shown be ubiquitous and important in soils, showing that *nirK*/*nirS* and *nosZ* ratios are much more complex than previously believed (Jones et al., 2014). Interestingly, our data agree with the laboratory study by Petersen et al. (2012), who found that *nosZ* clade I abundance is a good predictor of denitrification (**Figure 5** C1–3; correlation *nosZ*/N_2O $R = 0.61$, $p < 0.003$). While Petersen et al. (2012) based their findings on potential rates, our study corroborated the concept of *nosZ* as a bioindicator of denitrification with actual gas flux rates measured *in situ* at the same soil sampling time (Rocca et al., 2014). This is an interesting finding, since N_2O flux is a complex process involving other pathways, such as anammox (Butterbach-Bahl et al., 2013). Further studies should include other processes and soil types, to test why *nosZ* clade I is such

a good indicator for denitrification in some soils (Rocca et al., 2014).

In this context, the term 'denitrification regulatory phenotype' (DRP) has been proposed for the denitrification process, which includes a series of analyses of a community, even though all the players and processes are not completely understood (Bergaust et al., 2011). For instance, it encompasses the most important traits of the microbial activity in the denitrification process in the environment (Bergaust et al., 2011). Using this approach the most important parameters are measured (i.e., pH, NO_3^-, O_2, *nos*Z genes) and the overall results reported (i.e., N_2O and NO_x). This technique is particularly powerful for microcosm incubation, whereby soil samples can be transported from the field to the laboratory and incubated to measure the effect of O_2 and NO_3^- in the NO_x and N_2O fluxes of that particular microbial community, allowing a better understanding of denitrification in that soil sample. In this study we worked with measurements *in situ* that made these evaluations hard to evaluate under field conditions, and we suggest that this approach could generate very informative data for future work that could better explain the insights observed in our data.

The highest N_2O efflux from acidic forest soils and additional correlations with N concentrations, soil pH values, and total soil C were also observed in the present study (Richardson et al., 2009; Petersen et al., 2012). Paradoxically, even though land use change to agriculture affected the N-related microbial groups, it was beneficial in reducing potential nitrate leaching and N_2O emissions, mitigating environmental pollution and global warming (Wieder et al., 2013).

Methane Dynamics

Forest soils had the highest abundance of methanogens, however, pasture had the highest methane effluxes, showing that rather than looking for direct correlations between microorganisms and process rates, they need to be included in a multivariate framework (**Figure 3**). In pasture soils, cow grazing compacts the soil, resulting in more anaerobic sites, and consequently higher methane production (Frey et al., 2011; Šibanca et al., 2014), as detected by both the path analysis and multiple regressions (**Figure 3**). In addition, cow excrement, which is more easily degradable than forest litter, might serve as a nutrient source for soil microbes leading to increased methanogenic activity and probably also supplying methanogens to the soil (Gattinger et al., 2007; Prem et al., 2014). Methane dynamics are influenced by diffusional constraints on the net oxidation activity, which would be important to consider in future studies (Striegl, 1993).

The quantity of methanotrophs, represented by detection of the gene *pmo*A, correlated with the distribution of methanogens ($R = 0.82$, $p < 0.001$). Since the methanotrophs potentially consume the methane produced by methanogens, it is a plausible explanation for most of our observed data, as demonstrated by the path analysis (**Figure 3**). Additionally, Forests are known as net methane sinks, and methanotrophs are usually associated with this vegetation system (Conrad, 2002; Cerri et al., 2006). By the other hand, in the crop fields fungicides and pesticides can suppress methanotroph communities (Conrad, 2002). In fact, the *pmoA* gene abundance was correlated with total Bacterial abundance and their mutual suppression in soybean fields in the wet season is very likely related to crop management (e.g., pesticides and fungicides). We observed that after winter fallow the methanotroph abundances were more similar among the land uses (Supplementary Figure S2), showing that specific agricultural management during soybean growth affected these communities and that abundance of methanotrophs could be nearly restored after winter fallow.

Linking Gene Abundances, Soil Parameters, and Processes

Our data demonstrate that rather than searching for direct correlations between genes and biogeochemical processes, environmental factors, such as soil parameters, should also be included using regression analyses (**Figures 4** and **5**). Gene abundances have some correlation with processes, but the relationships of gene abundances with environmental factors are more important for analyzing the complex biogeochemical processes detailed here (Petersen et al., 2012; Levy-Booth et al., 2014). The net balance of chemicals in soil is a result of the interaction among the abundance and activities of microbial functional groups, the plants, and the soil physical and chemical characteristics (Levy-Booth et al., 2014). While climatic conditions, temperature and rain, can impact process rates within short time periods (hours–days) and soil characteristics (i.e., texture, total C) affect long-term predictions (seasons-years), gene abundances are important indicators for mid-term predictions (weeks–months) and for detailed microbial transformation dynamics and their effects on biogeochemical processes (Petersen et al., 2012; Levy-Booth et al., 2014).

Biogeochemical processes in soil are complex and our knowledge of the microbial players and controlling factors is constantly growing. New processes have been discovered in recent years (e.g., annamox) showing that our knowledge of the microorganisms and processes is still incomplete. In practice, no study to date has evaluated all the possible processes and variables in the C and N cycles, but instead focused on some of the main aspects (Petersen et al., 2012; Levy-Booth et al., 2014; Rocca et al., 2014). It follows that our paper does not attempt to evaluate all the possible aspects of the C and N cycles, but in studying some important aspects we bring advances to this research area.

For the first time we have detailed how the abundance of functional groups varied with land use change in soils of Southern Amazonia, and how these abundances are related to biogeochemical C and N cycles and GHG fluxes. We present integrated models of C and N cycles, showing gene abundances as key microbial indicators. Further studies with larger temporal and spatial surveys could help to explain how these parameters change through time and in different soils, and might also provide a database for predictive models. Also, improved primer design, additional marker genes (e.g., Jones et al., 2014; Levy-Booth et al., 2014), and expanded metadata for physico-chemical parameters, such as quantification of soil anaerobic micro-sites, and partitioning of SOM, could improve models and lead to a better understanding of the microbial regulation of biogeochemical processes in soils.

Metagenomic sequencing, which is primer independent, might be a more promising tool for true quantification of gene abundances in soil samples. However, cost is still a limiting factor. For example, while sequence analysis of a metagenomic library with minimum coverage costs around 2,000 dollars per sample, qPCR analysis costs around three dollars per gene within a sample (approximate prices accessed by the authors in 2014). Therefore, metagenomic sequencing can provide taxonomic and phylogenetic information, and thus both the vicinity of functional genes within other genomic content as well as material for better primer design. And qPCR is still a less expensive and powerful technique for gene abundance measurements, which allows integration of representative microbiological parameters into models of the C and N cycles. It enables broader surveys to analyze a higher number of samples, a requisite for modeling approaches.

Conclusion

The abundance of microbial functional groups, as determined by appropriate marker genes, fit well into associated biogeochemical process models, indicating the value of these data for a better mechanistic understanding of biogeochemical cycles. Land use change affected the abundance of select microbial groups in soils. Overall, forest soil contained a higher abundance of all marker genes targeting processes in the soil C and N cycle compared to other land uses. A seasonal trend was observed for the marker gene abundance, suggesting that land use effects were less significant after the dry winter fallow and more intense during the wet season and the associated crop growth. This means that agricultural management caused a disturbance in the growth or survival of functional groups that was restored almost to native levels after the dry winter fallow. We showed that integrating gene abundance with environmental parameters presented a better overview of biogeochemical processes. Further temporal and spatial surveys of the parameters discussed might help to improve our understanding of the ecological regulation of biogeochemical cycles, and should be incorporated into future predictive models.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01057

References

Ackerly, D. D., Thomas, W. W., Ferreira, C. A. C., and Pirani, J. R. (1989). The forest-cerrado transition zone in southern Amazonia: results of the 1985 Projeto Flora Amazônica expedition to Mato Grosso. *Brittonia* 41, 113–128. doi: 10.2307/2807515

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Bárta, J., Melichová, T., Vaněk, D., Picek, T., and Šantručková, H. (2010). Effect of pH and dissolved organic matter on the abundance of nirK and nirS denitrifiers in spruce forest soil. *Biogeochemistry* 101, 123–132. doi: 10.1007/s10533-010-9430-9

Bates, S. T., Berg-Lyons, D., Caporaso, J. G., Walters, W. A., Knight, R., and Fierer, N. (2011). Examining the global distribution of dominant archaeal populations in soil. *ISME J.* 5, 908–917. doi: 10.1038/ismej.2010.171

Bengtson, P., Sterngren, A. E., and Rousk, J. (2012). Archaeal abundance across a pH gradient in an arable soil and its relationship to bacterial and fungal growth rates. *Appl. Environ. Microbiol.* 78, 5906–5911. doi: 10.1128/AEM.01476-12

Bergaust, L., Bakken, L. R., and Frostegård, A. (2011). Denitrification regulatory phenotype, a new term for the characterization of denitrifying bacteria. *Biochem. Soc. Trans.* 39, 207–212. doi: 10.1042/BST0390207

Bouwman, A. F. (1998). Nitrogen oxides and tropical agriculture. *Nature* 392, 866–867. doi: 10.1038/31809

Brando, P. M., Nepstad, D. C., Davidson, E. A., Trumbore, S. E., Ray, D., and Camargo, P. (2008). Drought effects on litterfall, wood production and belowground carbon cycling in an Amazon forest: results of a throughfall reduction experiment. *Philos. Trans. R. Soc. B* 363, 1839–1848. doi: 10.1098/rstb.2007.0031

Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., and Conrad, R. (2007). Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. *Appl. Environ. Microbiol.* 73, 6876–6884. doi: 10.1128/AEM.01536-07

Bru, D., Ramette, A., Saby, N. P. A., Dequiedt, S., Ranjard, L., Jolivet, C., et al. (2011). Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. *ISME J.* 5, 532–542. doi: 10.1038/ismej.2010.130

Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., et al. (2013). Soil enzymes in a changing environment: current knowledge and future directions. *Soil Biol. Biochem.* 58, 216–234. doi: 10.1016/j.soilbio.2012.11.009

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., and Zechmeister-Boltenstern, S. (2013). Nitrous oxide emissions from soils, how well do we understand the processes and their controls. *Philos. Trans. R. Soc. Lond. B* 368, 16–21. doi: 10.1098/rstb.2013.0122

Cantarella, H., van Raij, B., and Quaggio, J. A. (1998). Soil and plant analyses for lime and fertilizer recommendations in Brazil. *Commun. Soil. Sci. Plant Anal.* 29, 11–14. doi: 10.1080/00103629809370060

Carvalho, J. L. N., Cerri, C. E. P., Cerri, C. C., Feigl, B. J., Piccolo, M. C., Godinho, V., et al. (2007). Changes of chemical properties in an Oxisol after clearing of native Cerrado vegetation for agricultural use in Vilhena. Rondonia State, Brazil. *Soil Tillage Res.* 96, 95–102. doi: 10.1016/j.still.2007.04.001

Carvalho, J. L. N., Raucci, G. S., Cerri, C. E. P., Bernoux, M., Feigl, B. J., Wruck, F. J., et al. (2010). Impact of pasture, agriculture and crop-livestock systems on soil C stocks in Brazil. *Soil Tillage Res.* 110, 175–186. doi: 10.1016/j.still.2010.07.011

Cerri, C. E. P., Piccolo, M. C., Feigl, B. J., Paustian, K., Cerri, C. C., Victoria, R. L., et al. (2006). Interrelationships among soil total C and N, trace gas fluxes,

microbial biomass, and internal N-cycling in soils under pasture of the Amazon region. *J. Sustain. Agric.* 27, 45–69. doi: 10.1300/J064v27n04_05

Claesson, M. J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, P., et al. (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic. Acids. Res.* 38:e200.

Conrad, R. (2002). Control of microbial methane production in wetland rice fields. *Nutr. Cycl. Agroecosyst.* 64, 59–69. doi: 10.1023/A:1021178713988

Dandie, C. E., Burton, D. L., Zebarth, B. J., Trevors, J. T., and Goyer, C. (2007). Analysis of denitrification genes and comparison of nosZ, cnorB and 16S rDNA from culturable denitrifying bacteria in potato cropping systems. *Syst. Appl. Microbiol.* 30, 128–138. doi: 10.1016/j.syapm.2006.05.002

Enwall, K., Throbäck, I. N., Stenberg, M., Söderström, M., and Hallin, S. (2010). Soil resources influence spatial patterns of denitrifying communities at scales compatible with land management. *Appl. Environ. Microbiol.* 76, 2243–2250. doi: 10.1128/AEM.02197-09

Fehr, W. R., Caviness, C. F., Burmood, D. T., and Pennington, J. S. (1971). Stage of development descriptions for soybeans, *Glycine max* (L.). *Merrill. Crop Sci.* 11, 929–931.

Food Agriculture Organization [FAO] (2014). *Food and Agriculture Organization of the United Nations*. Rome: Food Agriculture Organization. Available at: http://faostat.fao.org/site/339/default.aspx

Frey, B., Niklaus, P. A., Kremer, J., Lüscher, P., and Zimmermann, S. (2011). Heavy-machinery traffic impacts methane emissions as well as methanogen abundance and community structure in oxic forest soils. *Appl. Environ. Microbiol.* 77, 6060–6068. doi: 10.1128/AEM.05206-11

Galford, G., Melillo, J. M., Kicklighter, D. W., Cronin, T. W., Cerri, C. E. P., Mustard, J., et al. (2010). Greenhouse gas emissions from alternative futures of deforestation and agricultural management in the southern Amazon. *Proc. Natl. Aacd. Sci. U.S.A.* 107, 19649–11965. doi: 10.1073/pnas.10007 80107

Galford, G. L., Melillo, J. M., Kicklighter, D. W., Mustard, J. F., Cronin, T. W., Cerri, C. E. P., et al. (2011). Historical carbon emissions and uptake from the agricultural frontier of the Brazilian Amazon. *Ecol. Appl.* 21, 750–763. doi: 10.1890/09-1957.1

Gattinger, A., Höfle, M. G., Schloter, M., Embacher, A., Böhme, F., Munch, J. C., et al. (2007). Traditional cattle manure application determines abundance, diversity and activity of methanogenic Archaea in arable European soil. *Environ. Microb.* 9, 612–624. doi: 10.1111/j.1462-2920.2006.01181.x

Hallin, S., and Lindgren, P. E. (1999). PCR detection of genes encoding nitrite reductase in denitrifing bacteria. *Appl. Environ. Microbiol.* 65, 1652–1657.

Henry, S., and Bru, D. (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Appl. Environ. Microbiol.* 72, 5181–5189. doi: 10.1128/AEM.00231-06

Hou, Y., Zhang, H., Miranda, L., and Lin, S. (2010). Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal pcnA as the model gene. *PLoS ONE* 5:e9545. doi: 10.1371/journal.pone.0009545

Imfeld, G., and Vuilleumier, S. (2012). Measuring the effects of pesticides on bacterial communities in soil: a critical review. *Eur. J. Soil Biol.* 49, 22–30. doi: 10.1016/j.ejsobi.2011.11.010

Jiang, H., Huang, L., Deng, Y., Wang, S., Zhou, Y., Liu, L., et al. (2014). Latitudinal distribution of ammonia-oxidizing bacteria and archaea in the agricultural soils of eastern china. *Appl. Environ. Microbiol.* 80, 5593–5602. doi: 10.1128/AEM.01617-14

Jones, C. M., Spor, A., Brennan, F. P., Breuil, M.-C., Bru, D., Lemanceau, P., et al. (2014). Recently identified microbial guild mediates soil N_2O sink capacity. *Nat. Clim. Chang.* 4, 801–805. doi: 10.1038/nclimate2301

Jones, C. M., Stres, M., Rosenquist, M., and Hallin, S. (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Mol. Biol. Evol.* 25, 1955–1966. doi: 10.1093/molbev/msn146

Karlsson, A. E., Johansson, T., and Bengtson, P. (2012). Archaeal abundance in relation to root and fungal exudation rates. *FEMS Microbiol. Ecol.* 80, 305–311. doi: 10.1111/j.1574-6941.2012.01298.x

Kermekchiev, M. B., Kirilova, L. I., Vail, E. E., and Barnes, W. M. (2009). Mutants of Taq DNA Polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.* 37:e40. doi: 10.1093/nar/gkn1055

Lammel, D. R., Nüsslein, K., Tsai, S. M., and Cerri, C. C. (2015). Land use and soil chemistry drive bacterial community composition in samples from rainforest and Cerrado (Brazilian Savannah) biomes of Southern Amazonia. *Eur. J. Soil. Biol.* 66, 32–39. doi: 10.1016/j.ejsobi.2014.11.001

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., et al. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442, 806–809. doi: 10.1038/nature04983

Levy-Booth, D. J., Prescott, C. E., and Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biol. Biochem.* 75, 11–25. doi: 10.1128/AEM.01751-10

Maia, S. M. F., Ogle, S. M., Cerri, C. C., and Cerri, C. E. P. (2010). Changes in soil organic carbon storage under different agricultural management systems in the Southwest Amazon Region of Brazil. *Soil Tillage Res.* 106, 177–184. doi: 10.1016/j.still.2009.12.005

Mcdonald, I. R., Bodrossy, L., Chen, Y., and Murrell, J. C. (2008). Molecular ecology techniques for the study of aerobic methanotrophs. *App. Environ. Microbiol.* 74, 1305–1315. doi: 10.1128/AEM.02233-07

Meyer, A., Focks, A., Radl, V., Keil, D., Welzl, G., Schöning, I., et al. (2013). Different land use intensities in grassland ecosystems drive ecology of microbial communities involved in nitrogen turnover in soil. *PLoS ONE* 8:e73536. doi: 10.1371/journal.pone.0073536

Morales, S., Cosart, T., and Holben, W. (2010). Bacterial gene abundances as indicators of greenhouse gas emission in soils. *ISME J.* 4, 799–808. doi: 10.1038/ismej.2010.8

Neill, C., Melillo, J. M., Steudler, P. A., Cerri, C. C., Demoraes, J. F. L., Piccolo, M. C., et al. (1997). Soil carbon and nitrogen stocks following forest clearing for pasture in the southwestern Brazilian Amazon. *Ecol. Appl.* 7, 1216–1225. doi: 10.1890/1051-0761(1997)007[1216:SCANSF]2.0.CO;2

Pes, L. Z., Amado, T. J. C., Scala, N. L. Jr., Bayer, C., and Fiorind, J. E. (2011). The primary sources of carbon loss during the crop-establishment period in a subtropical Oxisol under contrasting tillage systems. *Soil Tillage* 117, 163–171. doi: 10.1016/j.still.2011.10.002

Petersen, D. G., Blazewicz, S. J., Firestone, M., Herman, D. J., Turetsky, M., and Waldrop, M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. *Environ. Microbiol.* 14, 993–1008. doi: 10.1111/j.1462-2920.2011.02679.x

Pinheiro, J. C., and Bates, D. M. (2000). *Mixed-Effects Models in S and S-PLUS*. Berlin: Springer.

Prem, E. M., Reitschuler, C., and Illmer, P. (2014). Livestock grazing on alpine soils causes changes in abiotic and biotic soil properties and thus in abundance and activity of microorganisms engaged in the methane cycle. *Europ. J. Soil. Biol.* 62, 22–29. doi: 10.1016/j.ejsobi.2014.02.014

Prosser, J. I., and Nicol, G. W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends Microbiol.* 20, 523–531. doi: 10.1016/j.tim.2012.08.001

R Core Team. (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.r-project.org

Richardson, D., Felgate, H., Watmough, N., Thomson, A., and Baggs, E. (2009). Mitigating release of the potent greenhouse gas N(2)O from the nitrogen cycle-could enzymic regulation hold the key? *Trends Biotechnol.* 27, 388–397. doi: 10.1016/j.tibtech.2009.03.009

Rocca, J. D., Lennon, J. T., and Evans, S. E. (2014). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. *ISME J.* 9, 1693–1699. doi: 10.1038/ismej.2014.252

Rösch, C., and Bothe, H. (2005). Improved assessment of denitrifying, N2-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. *Appl. Environ. Microbiol.* 71, 2026–2035.

Rosseel, Y. (2012). Lavaan: an R package for structural equation modeling. *J. Stat. Softw.* 48, 1–36.

Rotthauwe, J. H., Witze, K. P., and Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular

fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712.

Šibanca, N., Dumbrell, A. J., Mandic-Mule, I., and Maceka, I. (2014). Impacts of naturally elevated soil CO_2 concentrations on communities of soil archaea and bacteria. *Soil Biol. Biochem.* 68, 348–356. doi: 10.1016/j.soilbio.2013.10.018

Steinberg, L. M., and Regan, J. M. (2008). Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. *Appl. Environ. Microbiol.* 74, 6663–6671. doi: 10.1128/AEM.00553-08

Stempfhuber, B., Welzl, G., Wubet, T., Schöning, I., Marhan, S., Buscot, F., et al. (2014). Drivers for ammonia-oxidation along a land-use gradient in grassland soils. *Soil Biol. Biochem.* 69, 179–186. doi: 10.1016/j.soilbio.2013.11.007

Steudler, P. A., Bowden, R. D., Melillo, J. M., and Aber, J. D. (1989). Influence of nitrogen fertilization on methane uptake in temperate soils. *Nature* 341, 314–316. doi: 10.1038/341314a0

Stevenson, B. A., Hunter, D. W. F., and Rhodes, P. L. (2014). Temporal and seasonal change in microbial community structure of an undisturbed, disturbed, and carbon-amended pasture soil. *Soil Biol. Biochem.* 75, 175–185. doi: 10.1016/j.soilbio.2014.04.010

Striegl, R. G. (1993). Diffusional limits to the consumption of atmospheric methane by soils. *Chemosphere* 26, 715–720. doi: 10.1016/0045-6535(93)90455-E

Sugiyama, A., and Yazaki, K. (2012). "Root exudates of legume plants and their involvement in interactions with soil microbes," in *Secretions and Exudates in Biological Systems* eds J. M. Vivanco and F. Baluska (Berlin: Springer), 27–48. doi: 10.1007/s10886-013-0248-5

Tebbe, C. C., and Vahjen, W. (1993). Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *App. Environ. Microbiol.* 59, 2657–2665.

Throbäck, I. N., Enwall, K., Jarvis, A., and Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* 49, 401–417. doi: 10.1016/j.femsec.2004.04.011

Tripathi, B. M., Kim, M., Lai-Hoe, A., Shukor, N. A. A., Rahim, R. A., Go, R., et al. (2013). pH dominates variation in tropical soil archaeal diversity and community structure. *FEMS Microbiol. Ecol.* 86, 303–311. doi: 10.1111/1574-6941.12163

van Elsas, J. D., Duarte, G. F., Keijzer-Wolters, A., and Simit, S. (2000). Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. *J. Microbiol. Methods* 43, 133–151. doi: 10.1016/S0167-7012(00)00212-8

Verhamme, D. T., Prosser, J. I., and Nicol, G. W. (2011). Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *ISME J.* 5, 1067–1071. doi: 10.1038/ismej.2010.191

Vourlitis, G. L., Nogueira, J. S., Lobo, F. A. L., Sendall, K. M., Paulo, S. R., Dias, C. A., et al. (2008). Energy balance and canopy conductance of a tropical semi-deciduous forest of the southern Amazon Basin. *Water Resour. Res.* 44:W03412.

Wieder, W. R., Bonan, G. B., and Allison, S. D. (2013). Experimental removal and addition of leaf litter inputs reduces nitrate production and loss in a lowland tropical forest. *Biogeochemisry* 113, 629–642. doi: 10.1007/s10533-012-9793-1

Wood, S. N. (2006). *Generalized Additive Models: An Introduction with R.* Boca Raton, FL: Chapman and Hall/CRC.

Xia, Z., Xu, H., Chen, G., Dong, D., Bai, E., and Luo, L. (2013). Soil N2O production and the d15N–N2O value: their relationship with nitrifying/denitrifying bacteria and archaea during a growing season of soybean in northeast China. *Europ. J. Soil Biol.* 58, 73–80. doi: 10.1016/j.ejsobi.2013.05.008

Yao, H., He, Z., Wilson, M. J., and Campbell, C. D. (2000). Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. *Microb. Ecol.* 40, 223–237.

Yu, Y., Lee, C., Kim, J., and Hwang, S. (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol. Bioeng.* 89, 670–679. doi: 10.1002/bit.20347

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Differential Response of *Acidobacteria* Subgroups to Forest-to-Pasture Conversion and Their Biogeographic Patterns in the Western Brazilian Amazon

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Members of the phylum *Acidobacteria* are among the most abundant soil bacteria on Earth, but little is known about their response to environmental changes. We asked how the relative abundance and biogeographic patterning of this phylum and its subgroups responded to forest-to-pasture conversion in soils of the western Brazilian Amazon. Pyrosequencing of 16S rRNA genes was employed to assess the abundance and composition of the *Acidobacteria* community across 54 soil samples taken using a spatially nested sampling scheme at the landscape level. Numerically, *Acidobacteria* represented 20% of the total bacterial community in forest soils and 11% in pasture soils. Overall, 15 different *Acidobacteria* subgroups of the current 26 subgroups were detected, with *Acidobacteria* subgroups 1, 3, 5, and 6 accounting together for 87% of the total *Acidobacteria* community in forest soils and 75% in pasture soils. Concomitant with changes in soil chemistry after forest-to-pasture conversion—particularly an increase in properties linked to soil acidity and nutrient availability—we observed an increase in the relative abundances of *Acidobacteria* subgroups 4, 10, 17, and 18, and a decrease in the relative abundances of other *Acidobacteria* subgroups in pasture relative to forest soils. The composition of the total *Acidobacteria* community as well as the most abundant *Acidobacteria* subgroups (1, 3, 5, and 6) was significantly more similar in composition across space in pasture soils than in forest soils. These results suggest that preponderant responses of *Acidobacteria* subgroups, especially subgroups 1, 3, 4, 5, and 6, to forest-to-pasture conversion effects in soils could be used to define management-indicators of agricultural practices in the Amazon Basin. These acidobacterial responses are at least in part through alterations on acidity- and nutrient-related properties of the Amazon soils.

Keywords: tropical rainforest, land-use change, spatial scale, 16S rRNA gene, community similarity, *Acidobacteria*

INTRODUCTION

Land use change driven by human activities is considered the most important factor for biodiversity losses in the tropics (Sala et al., 2000) and a large number of studies have documented the negative effects of land use change for plants, animals (Gibson et al., 2011; Wearn et al., 2012), and most recently, microorganisms (Cenciani et al., 2009; Jesus et al., 2009; Navarrete et al., 2010, 2011, 2013, 2015; Taketani and Tsai, 2010; Rodrigues et al., 2013; Mirza et al., 2014; Mueller et al., 2014; Paula et al., 2014; Ranjan et al., 2015). For example, Rodrigues et al. (2013) reported that forest-to-pasture conversion resulted in a substantial decrease in the abundance of members of the bacterial phylum *Acidobacteria*.

Acidobacteria are among the most common bacteria in soils worldwide, including in Amazon soils (Kim et al., 2007; Jesus et al., 2009; Navarrete et al., 2010, 2013, 2015). The analysis of 16S rRNA gene sequences has demonstrated that acidobacterial abundance within a community may be regulated by soil pH (Fierer et al., 2007; Lauber et al., 2008; Jones et al., 2009; Rousk et al., 2010; Kuramae et al., 2011) and nutrient availability (Zhao et al., 2014). Genomic and physiological traits indicate characteristics that may contribute to *Acidobacteria* survival and growth in soil, such as the presence of membrane transporters and the ability to use carbon sources that span from simple sugars to more complex substrates such as hemicellulose, cellulose, and chitin; the reduction of nitrate, nitrite, and possibly nitric oxide; iron scavenging; and production of antimicrobial compounds (Ward et al., 2009; Rawat et al., 2012). In addition, Greening et al. (2015) proposed that consumption of trace gases such as H_2 provides a dependable general mechanism for *Acidobacteria* to generate maintenance energy required for long-term survival in soils.

Recently, increased attention has been paid to the response of *Acidobacteria* to environmental changes (George et al., 2009; Naether et al., 2012; Catão et al., 2014). Despite this appreciation for the phylum *Acidobacteria*, little is still known about the differential response at subgroup level to alterations in soil chemical properties and fertility, and how their community similarity change with distance in mosaic landscapes. Navarrete et al. (2013) reported the impact of agricultural management of soybean in Amazon forest soils on the composition of the *Acidobacteria* community, and they revealed that the abundance of *Acidobacteria* subgroups was related to soil chemical properties, which were clearly affected by agricultural management. These findings opened the possibility that subgroups of *Acidobacteria* could be used as management-indicators for the consequences of agricultural practices in the Amazon region.

The present study was designed to assess the *Acidobacteria* subgroup response at different geographic scales in primary forest and pasture soils. Firstly, we hypothesized that different subgroups of *Acidobacteria* respond differently to forest conversion into pastures in Amazon soils. Because of the substantial effects that land use change may have on soil chemical characteristics, we evaluated the differential response of *Acidobacteria* subgroups through the prism of the expected changes in soil chemical properties after forest-to-pasture conversion in the Amazon. In a corollary hypothesis, we tested whether taxonomic similarity of total *Acidobacteria* community and of their most abundant subgroups varies across space in forest and pasture soil samples in the western Brazilian Amazon. To address these hypotheses, we used pyrosequencing of the region V4 of the bacterial 16S rRNA gene to analyze the relative abundance and composition of the *Acidobacteria* community inhabiting soil from primary forests and pastures collected from the Amazon Rainforest Microbial Observatory, a model site representing the current expansive agricultural development of the region. We correlated the relative abundances of *Acidobacteria* at the taxonomic levels phylum and subgroup with soil chemical properties to explore group-specific responses to agricultural conversion. Furthermore, we explored the relationship between group-specific biogeographic patterns and land use change by comparing distance-decay relationship patterns.

MATERIALS AND METHODS

Site Description and Soil Sampling

This study was performed at the Fazenda Nova Vida (10°10′5″S and 62°49′27″W), located in the central region of the Brazilian state of Rondônia at the Amazon Rainforest Microbial Observatory (ARMO). Soils are classified as red-yellow podzolic latosol (Kandiudult). The climate is humid tropical, with an annual average temperature of 25.5°C and an average precipitation of 2200 mm (Bastos and Diniz, 1982). Local farmers employ slash-and-burn practices, i.e., clearing of primary forest followed by burning, in order to support livestock and farming systems in this region.

Soil samples were collected at the end of the rainy season (April 2009) from three primary forest sites and three pasture sites that had been continuously managed since 1987. At each site, a nested sampling scheme was established, centered on a 100 × 100 m (100 m^2) quadrat, with 10 × 10 m (10 m^2), and 1 × 1 m (1 m^2) quadrats nested within and adjacent to one corner of the 100 m^2 quadrat, for a total of nine sampling points per 100 m^2 quadrat (Figure S1). At each point, after the removal of the litter layer, the soil was sampled from 0 to 10 cm depth in the topsoil layer, gently homogenized, and subdivided. Samples were transported to the laboratory on ice. A portion of each sample was stored at −80°C for molecular analysis and another portion was stored at 4°C for soil chemical analysis.

Soil Chemical Properties and Statistical Analysis

The soil samples were dried and passed through a sieve (149 μm size). Total carbon (C) and nitrogen (N) were measured on a LECO CN elemental analyzer (St. Joseph, MI, USA) at the Soil Biogeochemistry Laboratory, Center for Nuclear Energy in Agriculture, University of São Paulo, Brazil. Soil chemical properties for each sample were analyzed at the Laboratory of Soil Fertility, Luiz de Queiroz College Agriculture, University of São Paulo, Brazil. Soil pH was measured from a soil/water (1:2.5)

suspension. Aluminum (Al), calcium (Ca), and magnesium (Mg) were extracted with 1 M potassium chloride. Ca and Mg were determined by atomic absorption spectrometry, while Al was determined by acid-base titration. Phosphorous (P) and potassium (K) were extracted by ion-exchange resin, and determined by colorimetry and atomic emission spectroscopy, respectively. Combined results were used for calculation of exchangeable bases (SB) as the sum of Ca, Mg, and K; cation-exchange capacity (CEC) as the sum of Ca, Mg, K, Al, and H; base saturation (V) as the percent relation between SB and CEC; aluminum saturation (m) as the percent relation between exchangeable Al and CEC; and potential acidity (H+Al), by an equation based on the pH determined in Shoemaker-McLean-Pratt (SMP) buffer solution. Analysis of similarity (ANOSIM) statistics was calculated to test for differences between forest and pasture soil chemical properties. A distance matrix (Euclidean metric) was constructed using non-transformed data. ANOSIM was carried out using Primer six (version 6.1.5, Primer-E Ltd., Plymouth, UK).

Isolation of DNA from Soil, Amplification, and Pyrosequencing of Bacterial 16S rRNA Genes

Total genomic DNA for each soil sample was extracted in triplicate using the Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The extractions for each sample were combined and DNA was quantified spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies, Inc., Wilmington, DE, USA). All DNA samples were stored at −20°C. The primer set 577F (5′-AYTGGGYDTAAAGNG-3′) and 926R (5′-CCGTCAATTCMTTTRAGT-3′) targeting the V4 region of bacterial 16S rRNA gene was used for the amplification. Group-specific primers for *Acidobacteria* such as Acid31F (Barns et al., 1999) and ACIDO (Lee and Cho, 2011) were not used in order to avoid the selective amplification and not detection of members of the phylum *Acidobacteria* such as 2, 22, and 25 as reported in many studies (Sait et al., 2006; Barns et al., 2007; George et al., 2009; Jones et al., 2009; Kielak et al., 2009; Lee and Cho, 2011). Adapter sequence was added to the primers as recommended by Roche (Table S1). Barcodes of 8 bp and AC linker were added to forward primers only. Each reaction was carried out in 50 μl reactions containing 1 × buffer, 1.8 mM of $MgCl_2$, 0.2 μM of each primer, 200 μM of deoxynucleoside triphosphate, 300 ng/μl of bovine serum albumin, 10 ng of DNA template and 1 μl of the enzyme FastStart High Fidelity PCR System (Roche Applied Sciences, Indianapolis, IN, USA), subjected to the following conditions: 95°C for 3 min; 30 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 1 min; and 72°C for 4 min. Each soil sample was amplified in triplicate, and reaction products were pooled and purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). PCR products were sequenced on a 454 GS FLX Sequencer (454 Life Sciences, Branford, CT, USA) at the Michigan State University Research Technology Support Facility. To prevent the possibility of sequencing errors (Huse et al., 2007), all reads were removed that either contained one or more ambiguous bases (N), had lengths outside the main distribution, or presented inexact matches to the primers used in the study. The high-quality bacterial 16S rRNA gene sequences are available through FigShare, http://dx.doi.org/10.6084/m9.figshare.1547935.

Sequence Analysis and Statistics

Sequences were processed using the bioinformatics platform QIIME version 1.7 (Caporaso et al., 2010). Sequences were removed from the analysis if they did not have the primer sequence, were less than 300 nt or more than 400 nt in length, contained a homopolymer run exceeding twenty nucleotides, or had ambiguous characters. The remaining sequences were assigned to samples by matching them to barcode sequences. Sequences that passed these quality filters were clustered into OTUs with a similarity cutoff of 97% using UCLUST (Edgar, 2010). Taxonomy was assigned to representative sequences from each OTU using the Ribosomal Database Project (Wang et al., 2007) web-based taxonomy assignment tool (http://rdp.cme.msu.edu/index.jsp) version 2.6 against the RDP 16S rRNA training set 9. The OTU table was filtered for specific taxonomic groups, and the relative abundance of *Acidobacteria* was estimated by comparing the number of sequences classified as belonging to the phylum with the number of classified bacterial sequences in each sample. Similarly, the relative abundance of *Acidobacteria* subgroups was estimated across all individual samples by comparing the number of sequences classified as belonging to each subgroup with the number of classified *Acidobacteria* sequences. Explicit relationships between the relative abundance of *Acidobacteria* subgroups and soil chemical properties were examined using constrained ordination generated by redundancy analysis (RDA) with the software CANOCO 4.5 (ter Braak and Šmilauer, 2002). Spearman's rank correlation coefficients were calculated between the relative abundance of *Acidobacteria* subgroups and soil properties using the "multtest" package (Pollard et al., 2005) in R (R Core Team, 2015). *P*-values were corrected for multiple testing, using the false discovery rate controlling procedure (Benjamini and Hochberg, 1995).

Distance-Decay of Similarity Analyses

The pairwise geographic distances between cores were calculated based on geographic coordinates and physical measurements. Community turnover (i.e., the distance-decay of similarity) was determined by regressing the pairwise community similarity against the pairwise logarithm of geographic distance using linear regression. Distance-decay slopes within taxonomic groups were compared between land types using the function diffslope in the software package "simba" (Jurasinski and Retzer, 2012) in R (R Core Team, 2015).

RESULTS

Soil Chemical Properties

Overall, statistical comparison of soil chemical properties for forest and pasture soils indicated that forest conversion to pasture

resulted in an increase in properties linked to soil acidity and nutrient availability in soil (Table S2). The chemical composition (Table S2) of forest and pasture soils differed significantly (ANOSIM, $R = 0.680$, $P = 0.002$). Potential acidity (H+Al) was significantly lower in forest soils compared to the pasture soils. Forest soils had significantly lower total C, N, S, and Mg contents and C/N ratios than pasture soils (Table S2).

Links between the Phylum *Acidobacteria*, Relative Abundances of Subgroup-Levels, and Soil Chemical Properties

The taxonomic analysis of the soil acidobacterial community was based on the retrieval of approximately 45,000 and 20,000 sequences of acidobacterial 16S rRNA gene fragments from forest soils and pasture soils, respectively (Table S3). The relative abundance of *Acidobacteria* sequences within an individual soil bacterial community represented on average 20% (±3.5%) in forest soil samples and 11% (±3.3%) in pasture soil samples. Overall, 15 different *Acidobacteria* subgroups of the current 26 subgroups (Hugenholtz et al., 1998; Zimmermann et al., 2005; Barns et al., 2007) were detected across the 54 soil samples, with *Acidobacteria* subgroups 1, 3, 5, and 6 accounting together for 87% of the total *Acidobacteria* community in forest soils and 75% in pasture soils (**Table 1**). A redundancy analysis of the relative abundance of *Acidobacteria* subgroups (1–7, 9–11, 13, 17, 18, 22, and 25) showed that the subgroups 1–3, 5, 9, 11, and 13 were significantly associated with forest soils while subgroups 4, 7, 10, 17, 18, and 25 were associated with pasture soils (**Figure 1**). *Acidobacteria* subgroup 6 was more related to pasture soils than forest soils. Statistically significant differences between forest vs. pasture soils were found for the relative abundances of the *Acidobacteria* subgroups 2 ($P < 0.005$), 4 ($P < 0.05$), 7 ($P < 0.0005$), 10 ($P < 0.05$), 13 ($P < 0.0005$), 17 ($P < 0.0005$) and 18 ($P < 0.005$) (**Table 1**). A correlation between the relative abundances of *Acidobacteria* subgroups and soil chemical properties revealed two distinct groups. *Acidobacteria* subgroups 1, 2, 3, and 13 were negatively correlated with total C and N content, C/N ratio, and P, S, K, Ca, and Mg content, and positively correlated with properties linked to soil acidity such as pH, Al, H+Al, and m; while subgroups 4, 5, 6, 7, 17, and 25 were positively correlated to nutrient availability and negatively correlated to properties linked to soil acidity (**Table 2**).

Acidobacterial Distance-Decay Relationships

Taxonomic similarity of the total *Acidobacteria* community was significantly correlated with geographic distance in both forest and pasture sites (**Table 3**). The slopes of the lines fitted to

TABLE 1 | Percentage of *Acidobacteria* subgroups relative to all *Acidobacteria* and of these to all *Bacteria* in forest and pasture sites.

	Forest sites			Pasture sites			Statistics
	F1	F2	F3	P1	P2	P3	F vs. P
Gp1	21.30 (11.6–28.5)[a]	9.67 (3.0–17.0)	32.76 (15.1–43.0)	13.02 (8.0–23.6)	26.17 (19.3–40.7)	12.34 (6.7–16.7)	ns[c]
Gp2	2.05 (0–4.4)	0.93 (0.3–2.0)	11.62 (2.4–22.3)	0.86 (0.5–2.0)	1.34 (0–3.6)	0.61 (0–1.4)	**
Gp3	29.31 (23.4–33.5)	15.80 (10.2–21.7)	24.73 (19.9–29.8)	16.8 (11.8–31.3)	19.06 (2.8–29.2)	19.59 (11.0–23.7)	ns
Gp4	3.18 (0–6.0)	5.31 (2.3–7.5)	1.67 (0.2–7.8)	4.29 (1.8–21.0)	4.76 (0.2–8.9)	12.55 (3.4–54.6)	*
Gp5	14.5 (9.6–18.6)	22.04 (16.7–30.8)	8.32 (4.8–14.5)	10.42 (8.9–26.5)	4.52 (1.7–9.0)	15.53 (8.9–24.9)	ns
Gp6	24.83 (16.8–33.7)	40.46 (30.1–51.6)	16.9 (4.6–35.4)	17.15 (19.7–37.4)	31.96 (20.4–44.0)	38.37 (27.8–45.7)	ns
Gp7	1.27 (0–3.0)	1.96 (0.2–3.8)	0.72 (0–1.7)	1.31 (0.6–4.0)	3.81 (2.0–5.5)	2.71 (1.8–5.0)	***
Gp9	0.02 (0–0.1)	0.31 (0–1.9)	ND[b]	0.01 (0–0.07)	ND	ND	ns
Gp10	0.06 (0.1–0.2)	0.12 (0–0.5)	0.13 (0.1–0.3)	0.1 (0–0.5)	0.23 (0–0.6)	0.25 (0–0.9)	*
Gp11	0.09 (0.2–0.5)	0.29 (0–1.2)	ND	0.01 (0–0.3)	0.01 (0–0.1)	0.04 (0–0.2)	ns
Gp13	1.80 (0.2–7.0)	0.55 (0–1.0)	2.3 (0.8–4.8)	0.34 (0–0.8)	0.49 (0–1.3)	0.26 (0–0.5)	***
Gp17	0.66 (0.3–1.8)	0.06 (0.03–1.9)	0.27 (0–0.3)	1.7 (1.3–5.4)	2.54 (0–3.6)	1.71 (1.2–2.7)	***
Gp18	0.05 (0.1–0.2)	0.05 (0–0.2)	0.01 (0–0.1)	0.13 (0–0.3)	0.43 (0–1.0)	0.08 (0–0.3)	**
Gp22	0.08 (0.2–0.3)	0.2 (0–1.0)	0.01 (0–0.05)	0.02 (0–0.3)	0.04 (0–0.2)	0.13 (0–0.3)	ns
Gp25	0.34 (0.2–0.6)	0.86 (0.4–1.4)	0,.05 (0–0.1)	0.05 (0–0.8)	0.97 (0–2.4)	0.93 (0.2–1.5	ns
unclassified *Acidobacteria*	0.23 (0.3–0.9)	0.36 (0–0.8)	0.2 (0–0.4)	0.09 (0–0.3)	0.04 (0–0.2)	0.28 (0–0.6)	ns
Total *Acidobacteria* community	17.51 (13.5–23.0)	20.1 (14.0–29.7)	24.15 (13.3–35.3)	7.26 (7.7–21.1)	14.3 (10.1–19.6)	11.08 (6.6–14.4)	ns

[a] *Average and range (%) of the average for each of nine replicate soils in each site.*

[b] *ND indicates that sequences of this subgroup were not detected. DNA sequences were classified into 26 acidobacterial subgroups using the Ribosomal Database Project 2 classifier (release 10.4). The 26 subgroups are classified according to the following designations: subgroups 1–8 according to Hugenholtz et al. (1998); subgroups 9–11 according to Zimmermann et al. (2005), and subgroups 12–26 according to Barns et al. (2007).*

[c] *Tukey's honestly significant difference (HSD) test was performed considering all pairwise comparisons between the 27 soil cores for forest sites and 27 soil cores for pasture sites. Significance levels: ns: $P > 0.05$, *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.*

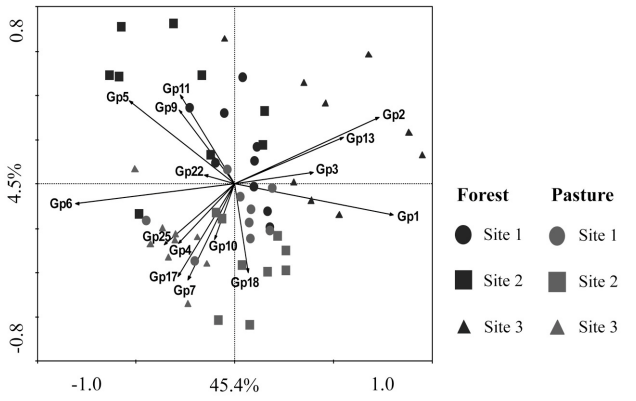

FIGURE 1 | Constrained ordination diagram for sample plots in the first two redundancy analysis (RDA) axes based on the soil chemical characteristics of the different sampling sites and their relationship with the relative abundance of *Acidobacteria* subgroups (1–7, 9–11, 13, 17, 18, 22, and 25). Each vector points to the direction of increase for a given *Acidobacteria* subgroup (Gp) and its length indicates the strength of the correlation between this variable and the ordination scores.

these relationships differed significantly between the forest and pasture soils with a significantly steeper slope for the total forest *Acidobacteria* community (**Figure 2**).

Taxonomic similarity was significantly correlated with distance for *Acidobacteria* subgroups 1, 3, 5, and 6 in both the forest and pasture soils (**Table 3**). For each group, forest distance-decay slopes were significantly steeper than their pasture counterparts (**Figure 2**). The distance-decay linear model showed a better fit to community similarity over distance for forest *Acidobacteria* communities than for those from pasture. Similar biogeographic patterns were revealed for the total *Acidobacteria* community and total bacterial community when comparing slopes across all forest and pasture soils (Figure S2).

DISCUSSION

The present study reports differential relative abundances for *Acidobacteria* at phylum and subgroup-levels in forest soils and in soils converted into pasture in the western Brazilian Amazon. These differences in abundances are correlated with soil acidity and nutrient availability. Total *Acidobacteria* community as well as the most abundant subgroups, namely 1, 3, 5, and 6, showed a divergence in spatial patterning between forest and pasture, with the pasture communities showing less spatial turnover than the forest communities.

Pasture establishment on acidic soils in the Amazon region is preceded by cutting and removing the economically important trees and burning the remaining above ground biomass (Fujisaka et al., 1996). As a result of these conversion and management practices, the thick organic layer of the forest is lost, the soil nutrient input is changed, and the topsoil is fertilized with alkaline ashes, thus increasing the soil pH (Juo and Manu, 1996; Giardina et al., 2000; Makeschin et al., 2008). Neye and Greenland (1960) proposed the "nutrient-rich ash" hypothesis to explain the observed short-term increase in soil nutrient availability after slash-and-burn clearing of forest. Although the slash-and-burn method of deforestation was applied 28 years before the soil sampling in our pasture sites, numerous studies of forest-to-pasture conversion in the Amazon reported increases in C and N stocks after several years of pasture establishment (Feigl et al., 1995; Neill et al., 1995, 1996; Cerri et al., 2004). Increases in C and N contents and nutrient availability in pasture soils can be also associated with a more decomposable litter (Rhoades et al., 2000; Potthast et al., 2010) and a dense fine-root system (Rhoades and Coleman, 1999) of the pasture grasses.

The chemical characteristics found in pasture soils can be a selective pressure for soil bacteria that prefer nutrient-rich habitats. Cultivation-dependent and -independent approaches have revealed adaptations of members of the phylum *Acidobacteria* to low substrate concentrations in soil, and their negative responses to increases in carbon and pH (Noll et al., 2005; Eichorst et al., 2007; Fierer et al., 2007; Ward et al., 2009). However, certain subgroups of the *Acidobacteria* are also known to have a preference for soil environments with increased available nutrients, i.e., copiotrophic environments (Navarrete et al., 2013). Despite the higher abundance of most *Acidobacteria* subgroups in forest soils, which may help to explain the strong decrease in the proportion of the total *Acidobacteria* community after forest-to-pasture conversion (Rodrigues et al., 2013), subgroups 7, 17, and 18 were significantly more abundant in pasture soils compared to the forest soils, with their abundances linked to high nutrient availability. *Acidobacteria* subgroup 7 showed similar response in soils from the Southeastern Brazilian Amazon converted into agricultural fields, with their abundances linked to high contents of nutrient in soil (Navarrete et al., 2013). Naether et al. (2012) also found higher relative abundances for members of *Acidobacteria* subgroups 17 in pasture soils in comparison to forest soils from three geographical regions in Germany. The selective advantage that allows microorganisms to respond rapidly in environments characterized by fluctuations in resource availability may be conferred by the number of rRNA gene copies in their genomes (Klappenbach et al., 2000; Stevenson and Schmidt, 2004). Genomes of *Acidobacteria* subgroups 1 and 3 were typified by a low number of rRNA gene copies (Ward et al., 2009). Although the number of rRNA gene copies is unknown for most of the *Acidobacteria* subgroups, the few number of ribossomal operons in acidobacterial genomes (Ward et al., 2009) is consistent with the higher abundance of this phylum in forest soils and has been postulated to be a characteristic marker of slow growth and a *K*-selected lifestyle (Klappenbach et al., 2000; Stevenson and Schmidt, 2004). Taken together, these findings suggest that different *Acidobacteria* subgroups have different life history patterns, with some preferring high nutrient concentrations and others preferring more oligotrophic environments.

The *Acidobacteria* subgroups 4 and 10 were also predominant in pasture soils and positively linked to soil pH. Previously, the abundance of the *Acidobacteria* subgroup 4 has been linked to increases in soil pH (Jones et al., 2009; Lauber et al., 2009). In

TABLE 2 | Spearman's rank correlation coefficients and statistical significance between abundance of *Acidobacteria* subgroups relative to all *Acidobacteria* and soil properties.

Soil properties	*Acidobacteria* subgroups														
	Gp1	Gp2	Gp3	Gp4	Gp5	Gp6	Gp7	Gp9	Gp10	Gp11	Gp13	Gp17	Gp18	Gp22	Gp25
pH	−0.535***	−0.419**	−0.302*	0.396**		0.529***					−0.398**				0.281*
N	−0.438***	−0.648***	−0.428***	0.571***		0.494***	0.455***				−0.599***	0.581***	0.307*		0.307*
C	−0.414***	−0.607***	−0.453***	0.549***		0.507***	0.515***				−0.611***	0.598***	0.362**		0.314*
C/N					−0.331**									−0.277*	
P	−0.678***	−0.446***	−0.256*	0.441***	0.455***	0.613***					−0.400**	0.263*			
S		−0.335*		0.290*							−0.294*	0.262*			0.367**
K	−0.514***	−0.324*		0.367**	0.303*	0.522***								0.265*	
Ca	−0.615***	−0.551***	−0.292*	0.570***	0.535***	0.518***		0.450***		0.291*	−0.465***	0.262*			0.271*
Mg	−0.393***	−0.494***		0.474***	0.290*	0.364**					−0.425***	0.354**			0.336*
Al	0.574***	0.478***		−0.431***	−0.400**	−0.496***		−0.300*	0.279*	−0.353**	0.448***				
H+Al	0.281*						0.312*						0.296*		
CEC	−0.390***	−0.540***	−0.414**	0.409**	0.310*	0.425***		0.365**			−0.422***	0.258*	0.256*		0.417**
V	−0.649***	−0.445***	0.544***		0.518***	0.511***		0.400**		0.316*	−0.363**				
m	0.644***	0.576***		−0.596***	−0.505***	−0.551***		−0.436***		−0.351*	0.449***	−0.280*			−0.311*

*Significance levels for the Spearman's rank coefficients are indicated at the *P < 0.05, **P < 0.005, ***P < 0.0005 levels. H+Al, potential acidity; CEC, cation exchange capacity; V, base saturation index; m, Al saturation index. Reference units are explained in Supplementary Table S2.*

TABLE 3 | Correlations of taxonomic similarity (Bray Curtis) and geographic distance of phylum *Acidobacteria* and subgroups with comparison of slope of linear model between land use types.

	Forest mantel R	Pasture mantel R	Difference in slope
Total *Acidobacteria* community	0.4133***	0.1369**	−0.01419***
Gp1	0.454***	0.255***	−0.02049***
Gp3	0.3074***	0.096*	−0.01125***
Gp5	0.636***	0.198***	−0.02518***
Gp6	0.1835**	0.1462**	−0.004582**

*Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001.*

addition, *Blastocatella fastidiosa*, the only known isolate from *Acidobacteria* subgroup 4, recovered from a savanna soil with a moderate acidic pH (i.e., close to 6.0) in Namibia, grows at even higher pHs (up to 10.0) (Foesel et al., 2013). Although soil pH has been demonstrated to explain a significant degree of microbial community variation in different spatial scales (Lauber et al., 2009), few studies have characterized the specific effects of pH on rare *Acidobacteria* subgroups in soil.

A large fraction of the total *Acidobacteria* community was composed of members of subgroup 1 in both forest and pasture soils. Sait et al. (2006) identified moderately acidic pH values as an important factor driving the abundance of members of this *Acidobacteria* subgroup in different soils, with *Acidobacteria* subgroup 1 increasing in relative abundance as the soil pH decreases. Li et al. (2014) showed significant negative correlations between *Acidobacteria* subgroup 1 and pH, and a positive correlation with C/N ratio. Rawat et al. (2013, 2014) and Ward et al. (2009) reported that members of *Acidobacteria* subgroup 1 are versatile heterotrophs that hydrolyze a suite of sugars and complex polysaccharides, contributing to carbon availability in certain ecosystems, including oligotrophic environments. This consideration was based on genomic data from *Granulicella mallensis* MP5ACTX8^T and *Granulicella tundricola* type strain MP5ACTX9^T, members of *Acidobacteria* subgroup 1 from tundra soil, and two acidobacterial subgroup 1 strains (*Acidobacterium capsulatum*), isolated from sediments in acidic drainage from the Yanahara pyrite mine in Japan. Isolation, cultivation and genome analysis of *Acidobacteria* subgroup 1 community members has revealed sugars as their preferred growth substrates (Männistö et al., 2011), and metabolic versatility with genes involved in metabolism and transport of carbohydrates, utilization and biosynthesis of diverse structural and storage polysaccharides such as plant based carbon polymers (Rawat et al., 2014).

The spatial turnover of a community (i.e., the rate of the distance-decay relationship) has been used as a proxy to estimate biotic homogenization at the landscape scale (Olden and Poff, 2003; Rodrigues et al., 2013). Through our approach we were able to detect changes to the spatial patterning of the *Acidobacteria* community as well as the most abundant *Acidobacteria* subgroups. In all cases, the directionality of change was the same; forest communities showed a steeper

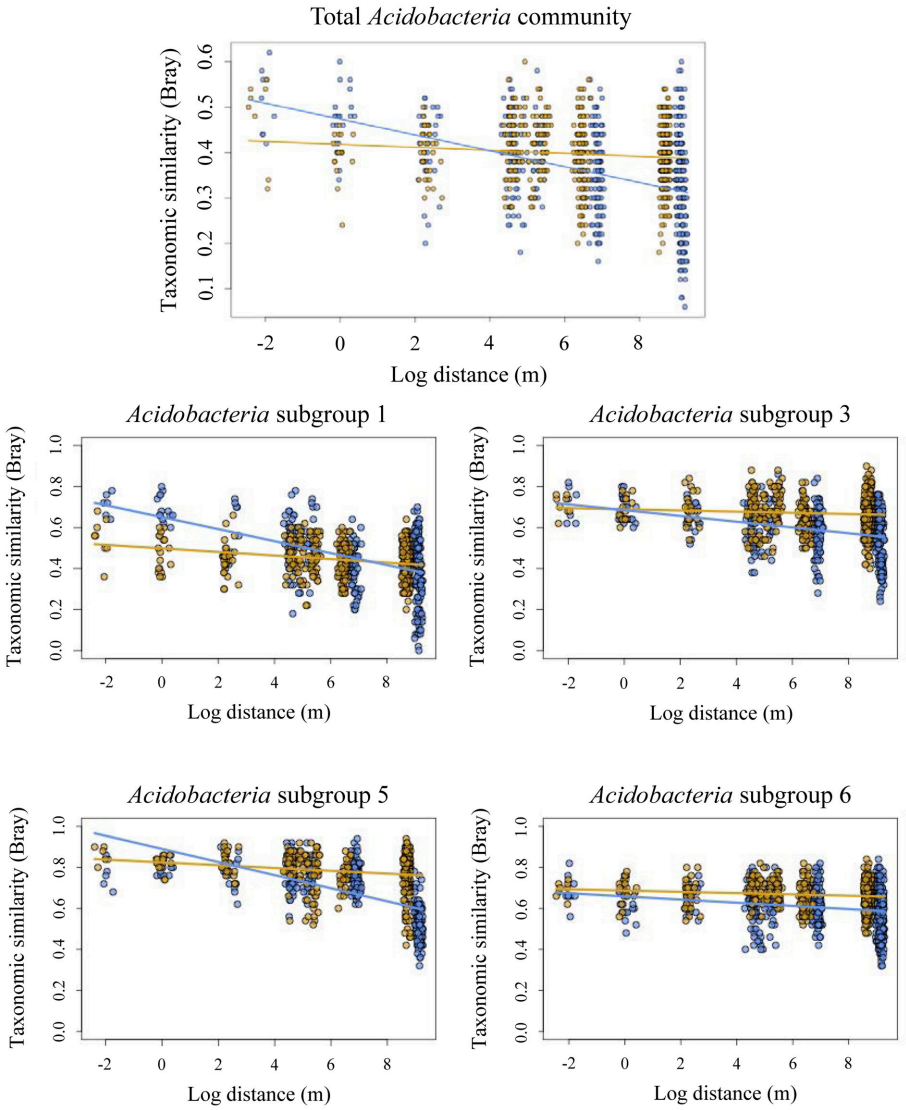

FIGURE 2 | Decay of taxonomic similarity (Bray–Curtis) with geographic distance in forest (blue) and pasture (yellow) for total acidobacterial community and *Acidobacteria* subgroups 1, 3, 5, and 6.

distance-decay relationship relative to pasture communities and that pasture communities were more similar to each other at larger distances than forest communities. We take these patterns to be suggestive of biotic homogenization. Changes to distance-decay patterns could result from alterations to several community assembly processes. For example, forest soils may have a more diverse or spatially variable array of microbial niches that may get broken down through the change in aboveground plant communities or alterations to the soil environment associated with land use change. It has been shown that *Acidobacteria* are one of the most abundant members of the phyllosphere of tropical trees, and that the distribution of *Acidobacteria* follows host plant phylogeny (Kim et al., 2012). Hence the removal and subsequent replacement of the tree community by low diversity grassland could be a strong driver in the changes to *Acidobacteria* biogeography.

These differential responses in relative abundance and biogeographic patterning of the *Acidobacteria* phylum and its subgroups to forest conversion into pastures in the Amazon rainforest expand the known possibilities to explore these subgroups to define management-indicators of agricultural practices. When conditions related to specific soil properties change owing to soil management practices, the proportion of different subgroups may be used to as an indicator of the soil status (Holt and Miller, 2011; Kuramae et al., 2011).

In conclusion, this study expands the understanding of ecological characteristics of *Acidobacteria* subgroups in Amazon soils by reporting differential responses of *Acidobacteria* and their

subgroups to forest-to-pasture conversion and the associated biogeographic patterns in a western Brazilian Amazon area. The forest clear-cutting and burning in the Amazon primarily to yield cattle pastures play a role in the assembly of the *Acidobacteria* communities in soil, especially in *Acidobacteria* subgroups 1, 3, 4, 5, and 6. Preponderant responses of *Acidobacteria* subgroups to forest-to-pasture conversion effects in soils are at least in part through effects on soil acidity and nutrient availability. The results also showed more similar composition of the total *Acidobacteria* community as well as the most abundant *Acidobacteria* subgroups across space in pasture soils than in forest soils. Taken together, these findings could assist to define management-indicators to judge the impacts from the forest-to-pasture conversion on soil ecosystem in the Amazon Basin.

AUTHOR CONTRIBUTIONS

AN and JR designed research; AN, AV, KM, AK, JT, KN, BB, ST, and JR performed research; JT, BB, ST, KN, and JR contributed new reagents/analytic tools; AN, AV, KM, AK, and JR analyzed data; and AN, KM, AK, BB, KN, and JR wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01443

REFERENCES

Barns, S. M., Cain, E. C., Sommerville, L., and Kuske, C. R. (2007). *Acidobacteria* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. *Appl. Environ. Microbiol.* 73, 3113–3116. doi: 10.1128/AEM.02012-06

Barns, S. M., Takala, S. L., and Kuske, C. R. (1999). Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. *Appl. Environ. Microbiol.* 65, 1731–1737.

Bastos, T. X., and Diniz, T. D. (1982). *Avaliação do Clima do Estado de Rondônia Para Desenvolvimento Agrícola*. Belém: Embrapa-CPATU.

Benjamini, Y., and Hochberg, Y. (1995). Controling the false discovery rate - a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B Stat. Methodol.* 57, 289–300.

ter Braak, C. J. F., Šmilauer, P. (2002). *CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5)*. New York, NY: Microcomputer Power.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Catão, E. C. P., Lopes, F. A. C., Araújo, J. F., Castro, A. P., Barreto, C. C., Bustamante, M. M. C., et al. (2014). Soil acidobacterial 16S rRNA gene sequences reveal subgroup level differences between savanna-like Cerrado and Atlantic forest brazilian biomes. *Int. J. Microbiol.* 2014:156341. doi: 10.1155/2014/156341

Cenciani, K., Lambais, M. R., Cerri, C. C., De Azevedo, L. C. B., and Feigl, B. J. (2009). Bacteria diversity and microbial biomass in forest, pasture and fallow soils in the southwestern Amazon Basin. *Rev. Bras. Cienc. Solo* 33, 907–916. doi: 10.1590/S0100-06832009000400015

Cerri, C. E. P., Paustian, K., Bernoux, M., Victoria, R. L., Melillos, J. M., and Cerri, C. C. (2004). Modeling changes in soil organic matter in Amazon forest to pasture conversion with the Century model. *Glob. Change Biol.* 10, 815–832. doi: 10.1111/j.1365-2486.2004.00759.x

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Eichorst, S. A., Breznak, J. A., and Schmidt, T. M. (2007). Isolation and characterization of soil bacteria that define Terriglobus gen. nov., in the phylum Acidobacteria. *Appl. Environ. Microb.* 73, 2708–2717. doi: 10.1128/AEM.02140-06

Feigl, B. J., Melillo, J. M., and Cerri, C. C. (1995). Changes in the origin and the quality of soil organic matter after pasture introduction in Rondônia (Brazil). *Plant Soil* 175, 21–29. doi: 10.1007/BF02413007

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology* 88, 135–1364. doi: 10.1890/05-1839

Foesel, B. U., Rohde, M., and Overmann, J. (2013). Blastocatella fastidiosa gen. nov., sp. nov., isolated from semiarid savanna soil - the first described species of Acidobacteria subdivision 4. *Syst. Appl. Microbiol.* 36, 82–89. doi: 10.1016/j.syapm.2012.11.002

Fujisaka, S., Bell, W., Thomas, N., Hurtad, L., and Crawford, E. (1996). Slash-and-burn agriculture, conversion to pasture, and deforestation in two Brazilian Amazon colonies. *Agr. Ecosyst. Environ.* 59, 115–130. doi: 10.1016/0167-8809(96)01015-8

George, I. F., Liles, M. R., Hartmann, M., Ludwig, W., Goodman, R. M., and Agathos, S. N. (2009). Changes in soil Acidobacteria communities after 2,4,6-trinitrotoluene contamination. *FEMS Microbiol. Lett.* 296, 159–166. doi: 10.1111/j.1574-6968.2009.01632.x

Giardina, C. P., Sanford, R. L., Døckersmith, I. C., and Jaramillo, V. J. (2000). The effects of slash burning on ecosystem nutrients during the land preparation phase of shifting cultivation. *Plant Soil* 220, 247–260. doi: 10.1023/A:1004741125636

Gibson, L., Lee, T. M., Koh, L. P., Brook, B. W., Gardner, T. A., Barlow, J., et al. (2011). Primary forests are irreplaceable for sustaining tropical biodiversity. *Nature* 478, 378–381. doi: 10.1038/nature10425

Greening, C., Carere, C. R., Rushton-Green, R., Harold, L. K., Hards, K., Taylor, M. C., et al. (2015). Persistence of the dominant soil phylum *Acidobacteria* by trace gas scavenging. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10497–10502. doi: 10.1073/pnas.1508385112

Holt, E. A., and Miller, S. W. (2011). Bioindicators: using organisms to measure environmental impacts. *Nat. Educ. Knowl.* 3, 8.

Hugenholtz, P., Goebel, B. M., and Pace, N. R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.

Huse, S. M., Huber, J. A., Morrison, H. G., Sogin, M. L., and Welch, D. M. (2007). Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biol.* 8, R143. doi: 10.1186/gb-2007-8-7-r143

Jesus, E. C. D., Marsh, T. L., Tiedje, J. M., and Moreira, F. M. S. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Jones, R. T., Robeson, M. S., Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J.* 3, 442–453. doi: 10.1038/ismej.2008.127

Juo, A. S. R., and Manu, A. (1996). Chemical dynamics in slash-and-burn agriculture. *Agric. Ecosyst. Environ.* 58, 49–60. doi: 10.1016/0167-8809(95)00656-7

Jurasinski, G., and Retzer, V. (2012). *Simba: A Collection of Functions for Similarity Analysis of Vegetation Data. R package Version 0.3-5.* Available online at: http://CRAN.R-project.org/package=simba

Kielak, A., Pijl, A. S., van Veen, J. A., and Kowalchuk, G. A. (2009). Phylogenetic diversity of Acidobacteria in a former agricultural soil. *ISME J.* 3, 378–382. doi: 10.1038/ismej.2008.113

Kim, J. S., Sparovek, G., Longo, R. M., De Melo, W. J., and Crowley, D. (2007). Bacteria diversity of terra preta and pristine forest soil from the western Amazon. *Soil Biol. Biochem.* 39, 684–690. doi: 10.1016/j.soilbio.2006.08.010

Kim, M., Singh, D., Lai-Hoe, A., Go, R., Abdul Rahim, R., Ainuddin, A. N., et al. (2012). Distinctive phyllosphere bacterial communities in tropical trees. *Microb. Ecol.* 63, 674–681. doi: 10.1007/s00248-011-9953-1

Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of Bacteria. *Appl. Environ. Microbiol.* 66, 1328–1333. doi: 10.1128/AEM.66.4.1328-1333.2000

Kuramae, E. E., Yergeau, E., Wong, L. C., Pijl, A. S., van Veen, J. A., and Kowalchuk, G. A. (2011). Soil characteristics more strongly influence soil bacterial communities than land-use type. *FEMS Microbiol. Ecol.* 79, 12–24. doi: 10.1111/j.1574-6941.2011.01192.x

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community composition at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi: 10.1128/AEM.00335-09

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40, 2407–2415. doi: 10.1016/j.soilbio.2008.05.021

Lee, S.-H., and Cho, J.-C. (2011). Group-specific PCR primers for the phylum Acidobacteria designed based on the comparative analysis of 16S rRNA gene sequences. *J. Microbiol. Methods* 86, 195–203. doi: 10.1016/j.mimet.2011.05.003

Li, H., Ye, D., Wang, X., Settles, M. L., Wang, J., Hao, Z., et al. (2014). Soil bacterial communities of different natural forest types in Northeast China. *Plant Soil* 383, 203–216. doi: 10.1007/s11104-014-2165-y

Makeschin, F., Haubrich, F., Abiy, M., Burneo, J. I., and Klinger, T. (2008). "Pasture management and natural soil regeneration," in *Gradients in a Tropical Mountain Ecosystem of Ecuador - Ecological Studies*, eds E. Beck, J. Bendix, I. Kottke, F. Makeschin, and R. Mosandl (Berlin: Springer), 397–408. doi: 10.1007/978-3-540-73526-7_38

Männistö, M. K., Rawat, S., Starovoytov, V., and Häggblom, M. M. (2011). Terriglobus saanensis sp. nov., a novel Acidobacterium isolated from tundra soil of Northern Finland. *Int. J. Syst. Evol. Microbiol.* 61, 1823–1828. doi: 10.1099/ijs.0.026005-0

Mirza, B. S., Potisap, C., Nüsslein, K., Bohannan, B. J. M., and Rodrigues, J. L. M. (2014). Response of free-living nitrogen-fixing microorganisms to land use change in the Amazon rainforest. *Appl. Environ. Microbiol.* 80, 281–288. doi: 10.1128/AEM.02362-13

Mueller, R. C., Paula, F. S., Mirza, B. S., Rodrigues, J. L. M., Nüsslein, K., and Bohannan, B. J. M. (2014). Links between plant and fungal communities across a deforestation chronosequence in the Amazon rainforest. *ISME J.* 8, 1548–1550. doi: 10.1038/ismej.2013.253

Naether, A., Foesel, B. U., Naegele, V., Wüst, P. K., Weinert, J., Bonkowski, M., et al. (2012). Environmental factors affect acidobacterial communities below the subgroup level in grassland and forest soils. *Appl. Environ. Microbiol.* 78, 7398–7406. doi: 10.1128/AEM.01325-12

Navarrete, A. A., Cannavan, F. S., Taketani, R. G., and Tsai, S. M. (2010). A molecular survey of the diversity of microbial communities in different Amazonian agricultural model systems. *Diversity* 2, 787–809. doi: 10.3390/d2050787

Navarrete, A. A., Kuramae, E. E., de Hollander, M., Pijl, A. S., van Veen, J. A., and Tsai, S. M. (2013). Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiol. Ecol.* 83, 607–621. doi: 10.1111/1574-6941.12018

Navarrete, A. A., Taketani, R. G., Mendes, L. W., Cannavan, F. S., Moreira, F. M. S., and Tsai, S. M. (2011). Land-use systems affect archaeal community structure and functional diversity in western Amazon soils. *Rev. Bras. de Cienc. Solo* 35, 1527–1540. doi: 10.1590/s0100-06832011000500007

Navarrete, A. A., Tsai, S. M., Mendes, L. W., Faust, K., de Hollander, M., Cassman, N., et al. (2015). Soil microbiome responses to the short-term effects of Amazonian deforestation. *Mol. Ecol.* 24, 2433–2448. doi: 10.1111/mec.13172

Neill, C., Fry, B., Melillo, J. M., Steudler, P. A., Moraes, J. F. L., and Cerri, C. C. (1996). Forest and pasture derived carbon contributions to carbon stocks and microbial respiration of tropical pasture soils. *Oecologia* 107, 113–119. doi: 10.1007/BF00582241

Neill, C., Piccolo, M. C., Steudler, P. A., Melillo, J. M., Feigl, B. J., and Cerri, C. C. (1995). Nitrogen dynamics in soils of forests and active pastures in the western Brazilian Amazon basin. *Soil Biol. Biochem.* 27, 1167–1175. doi: 10.1016/0038-0717(95)00036-E

Neye, P., and Greenland, D. (1960). The soil under shifting cultivation. *Tech. Commun. Common Wealth Agric. Bur.* 51, 156.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382–395. doi: 10.1111/j.1462-2920.2005.00700.x

Olden, J. D., and Poff, N. L. (2003). Toward a mechanistic understanding and prediction of biotic homogenization. *Am. Nat.* 162, 442–460. doi: 10.1086/378212

Paula, F. S., Rodrigues, J. L. M., Zhou, J., Wu, L., Mueller, R. C., Mirza, B. S., et al. (2014). Land use change alters functional gene diversity, composition and abundance in Amazon forest soil microbial communities. *Mol. Ecol.* 23, 2988–2999. doi: 10.1111/mec.12786

Pollard, K. S., Dudoit, S., and van der Laan, M. J. (2005). "Multiple testing procedures: R multtest package and applications to genomics" in *Bioinformatics and computational biology solutions using R and bioconductor*, eds R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber (New York, NY: Springer), 251–272.

Potthast, K., Hamer, U., and Makeschin, F. (2010). Impact of litter quality on mineralization processes in managed and abandoned pasture soils in Southern Ecuador. *Soil Biol. Biochem.* 42, 56–64. doi: 10.1016/j.soilbio.2009.09.025

Ranjan, K., Paula, F. S., Mueller, R. C., Jesus, E. C., Cenciani, K., Bohannan, B. J., et al. (2015). Forest-to-pasture conversion increases the diversity of the phylum Verrucomicrobia in Amazon rainforest soils. *Front. Microbiol.* 6:779. doi: 10.3389/fmicb.2015.00779

Rawat, S. R., Männistö, M. K., Bromberg, Y., and Haggblom, M. M. (2012). Comparative genomic and physiological analysis provides insights into the role of *Acidobacteria* in organic carbon utilization in Arctic tundra soils. *FEMS Microbiol. Ecol.* 82, 341–355. doi: 10.1111/j.1574-6941.2012.01381.x

Rawat, S. R., Männistö, M. K., Starovoytov, V., Goodwin, L., Nolan, M., Hauser, L., et al. (2014). Complete genome sequence of *Granulicella tundricola* type strain MP5ACTX9T, an *Acidobacteria* from tundra soil. *Stan. Genomic Sci.* 9, 449–461. doi: 10.4056/sigs.4648353

Rawat, S. R., Männistö, M. K., Starovoytov, V., Goodwin, L., Nolan, M., Hauser, L. J., et al. (2013). Complete genome sequence of *Granulicella mallensis* type strain MP5ACTX8T, an *acidobacterium* from tundra soil. *Stand. Genomic Sci.* 9, 71–82. doi: 10.4056/sigs.4328031

R Core Team (2015). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Vienna. Available online at: http://www.R-project.org/

Rhoades, C. C., and Coleman, D. C. (1999). Nitrogen mineralization and nitrification following land conversion in montane Ecuador. *Soil Biol. Biochem.* 31, 1347–1354. doi: 10.1016/S0038-0717(99)00037-1

Rhoades, C. C., Eckert, G. E., and Coleman, D. C. (2000). Soil carbon differences among forest, agriculture, and secondary vegetation in lower montane Ecuador. *Ecol. Appl.* 10, 497–505. doi: 10.1890/1051-0761(2000)010[0497:SCDAFA]2.0.CO;2

Rodrigues, J. L. M., Pellizari, V. H., Mueller, R., Baek, K., Jesus, E. C., Paula, F. S., et al. (2013). Conversion of the Amazon rainforest to agriculture results in biotic homogenization of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 110, 988–993. doi: 10.1073/pnas.1220608110

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., et al. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* 4, 1340–1351. doi: 10.1038/ismej.2010.58

Sait, M., Davis, K. E., and Janssen, P. H. (2006). Effect of pH on isolation and distribution of members of subdivision 1 of the phylum *Acidobacteria* occurring in soil. *Appl. Environ. Microbiol.* 72, 1852–1857. doi: 10.1128/AEM.72.3.1852-1857.2006

Sala, O. E., Chapin, F. S., Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., et al. (2000). Biodiversity - global biodiversity scenarios for the year 2100. *Science* 287, 1770–1774. doi: 10.1126/science.287.5459.1770

Stevenson, B. S., and Schmidt, T. M. (2004). Life history implications of rRNA gene copy number in *Escherichia coli*. *Appl. Environ. Microbiol.* 70, 6670–6677. doi: 10.1128/AEM.70.11.6670-6677.2004

Taketani, R. G., and Tsai, S. M. (2010). The influence of different land uses on the structure of archaeal communities in Amazonian anthrosols based on 16S rRNA and *amo*A genes. *Microb. Ecol.* 59, 734–743. doi: 10.1007/s00248-010-9638-1

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M., Wu, M., et al. (2009). Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Appl. Environ. Microb.* 75, 2046–2056. doi: 10.1128/AEM.02294-08

Wearn, O. R., Reuman, D. C., and Ewers, R. M. (2012). Extinction debt and windows of conservation opportunity in the Brazilian Amazon. *Science* 337, 228–232. doi: 10.1126/science.1219013

Zhao, J., Ni, T., Li, Y., Xiong, W., Ran, W., Shen, B., et al. (2014). Responses of bacterial communities in arable soils in a rice-wheat cropping system to different fertilizer regimes and sampling times. *PLoS ONE* 9:e85301. doi: 10.1371/journal.pone.0085301

Zimmermann, J., Gonzalez, J. M., Saiz-Jimenez, C., and Ludwig, W. (2005). Detection and phylogenetic relationships of highly diverse uncultured acidobacterial communities in altamira cave using 23S rRNA sequence analyses. *Geomicrobiol. J.* 22, 379–388. doi: 10.1080/01490450500248986

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Forest-to-pasture conversion increases the diversity of the phylum *Verrucomicrobia* in Amazon rainforest soils

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The Amazon rainforest is well known for its rich plant and animal diversity, but its bacterial diversity is virtually unexplored. Due to ongoing and widespread deforestation followed by conversion to agriculture, there is an urgent need to quantify the soil biological diversity within this tropical ecosystem. Given the abundance of the phylum *Verrucomicrobia* in soils, we targeted this group to examine its response to forest-to-pasture conversion. Both taxonomic and phylogenetic diversities were higher for pasture in comparison to primary and secondary forests. The community composition of *Verrucomicrobia* in pasture soils was significantly different from those of forests, with a 11.6% increase in the number of sequences belonging to subphylum 3 and a proportional decrease in sequences belonging to the class *Spartobacteria*. Based on 99% operational taxonomic unit identity, 40% of the sequences have not been detected in previous studies, underscoring the limited knowledge regarding the diversity of microorganisms in tropical ecosystems. The abundance of *Verrucomicrobia*, measured with quantitative PCR, was strongly correlated with soil C content ($r = 0.80$, $P = 0.0016$), indicating their importance in metabolizing plant-derived carbon compounds in soils.

Keywords: tropical forest, microbial biodiversity, land use change, habitat filtering, biotic homogenization

Introduction

The Amazon rainforest is the largest equatorial forest in the world, encompassing 40% of the world's tropical ecosystems (Laurance et al., 2001). It maintains one-fifth of the world's total freshwater volume, influencing hydrological and climatological cycles and balancing the global flux of atmospheric gasses. The Amazon rainforest harbors an estimated 40,000 vascular plant species, 5,500 vertebrate species, and 100,000 invertebrate species (Da Silva et al., 2005; Lewinsohn and Prado, 2005). The Amazon rainforest is under constant threat from agricultural conversion, with approximately 16% of the original cover already lost (Soares-Filho et al., 2006). Land use change driven by human activities is considered the most important factor for biodiversity losses in the tropics (Sala et al., 2000) and a large number of studies have documented the negative effects of land use change on plants and animals (Gibson et al., 2011; Wearn et al., 2012).

Recently, it has been shown that forest-to-pasture conversion also impacts microorganisms, resulting in biotic homogenization of communities (Rodrigues et al., 2013). Specifically, there was a

substantial decrease in the abundance of *Acidobacteria* 16S rRNA gene sequences and correspondent increase in sequences identified as belonging to the phyla *Firmicutes*, *Actinobacteria,* and *Chloroflexi.* Other phyla did not show an apparent response to conversion, including the *Verrucomicrobia;* however, this group has exhibited strong responses to land use change in previous studies (Kielak et al., 2008; Suleiman et al., 2013; Montecchia et al., 2015; Prober et al., 2015) and it is possible that shifts within the *Verrucomicrobia* were masked by limited coverage within the overall bacterial community (Bergmann et al., 2011).

The *Verrucomicrobia* is a ubiquitous and abundant group in many terrestrial ecosystems (Buckley and Schmidt, 2001; Janssen, 2006; Bergmann et al., 2011; Fierer et al., 2013). The ecology of this group is unclear, but they have potential roles in methane oxidation and polysaccharide degradation (Martinez-Garcia et al., 2012), and the high relative abundance of this group in soils across a wide range of ecosystems suggests that they play important, but poorly understood, roles. Members of the *Verrucomicrobia* are difficult to isolate and, to date, only a limited number of isolates (total of 31) have been obtained from soil samples (RDP II)[1], with four out of seven classes remaining without representatives in culture (Hedlund, 2011). The abundance and class composition of this phylum remain virtually unknown in tropical forest, and it is unclear to what extent members of this phylum can recover from agricultural conversion when pastures are abandoned and secondary forests are established.

In this study, we sought to fill the knowledge gap regarding the abundance and class-level distribution of *Verrucomicrobia* in the Amazon rainforest by addressing the following questions: (i) How is the diversity and community composition of *Verrucomicrobia* in Amazon rainforest soils affected by deforestation? and (ii) What are the environmental variables associated with the presence of *Verrucomicrobia* in Amazon rainforest soils? We hypothesized that the abundance and diversity of this phylum would be higher in the primary forest than in adjacent pasture and secondary forest sites. In order to answer the above questions, we designed and tested a specific oligonucleotide PCR primer for the detection of the phylum *Verrucomicrobia,* determined the effects of forest-to-pasture conversion on their community structure, and correlated the presence of members of *Verrucomicrobia* to local environmental conditions.

Materials and Methods

Site Description, Soil Collection, and Physicochemical Analyses

Soil samples were collected at the Fazenda Nova Vida (10°10′5″S and 62°49′27″W) situated in the western Amazon Basin, state of Rondonia, Brazil, in February of 2004. The annual precipitation reaches 2,200 mm and the annual temperature averages 25.5°C at this site (Bastos and Diniz, 1982), with only two distinct seasons: dry and wet. Soils are classified as Ultisols (US soil taxonomy), representing 22% of the Brazilian Amazon basin (Moraes et al., 1995). This area experiences the highest rate of deforestation in the Amazon basin, driven in large part by conversion of forest into pasture for cattle production.

Three different land use types were selected for sampling: primary forest (hereafter referred to as forest), a pasture established in 1987, and a secondary forest resulting from pasture abandonment in 1994 and subsequent natural re-colonization by forest plants. The common procedure for pasture establishment is aerial seeding of two fast growing grasses *Urochloa brizantha* (Hochst. ex A. Rich; formely the genus *Brachiaria*) and *Panicum maximum* without the use of chemical fertilizers or agricultural machinery. The plant community composition of the secondary forest is a mix of woody species and grasses (Feigl et al., 2006). Twenty-five independent soil samples per treatment (0–10 cm) were collected 50 m apart from each other in order to cover the 3 ha area plots, kept on ice, and transported to an on-site laboratory, where they were randomly combined into groups of five and sieved with a 2 mm mesh. The resulting five replicates per treatment were stored at −80°C prior to molecular analysis (Cenciani et al., 2009).

The soil under all land uses in this study is classified as a red–yellow podzolic latosol (Kandiudult) with sandy loam texture (Feigl et al., 2006). Soil samples were sieved with a 2-mm mesh and used for total C and N determination with an Auto Analyzer LECO TruSpec CN at the Centro de Energia Nuclear na Agricultura, University of Sao Paulo, Brazil. The soil attributes pH, organic matter, base saturation, cation exchange capacity, Al^{+} saturation, C/N, moisture, potential acidity, and elemental analysis of P, S, K^{+}, Ca^{+2}, Mg^{+2}, Al^{+3}, H^{+}, B, Cu, Fe, Mn, Zn were analyzed at the Department of Soil Sciences, University of Sao Paulo, Brazil, as previously described (Cenciani et al., 2009).

Verrucomicrobia Primer Design and Evaluation

A *Verrucomicrobia*-specific 16S rRNA gene-targeted primer was designed using the ARB software package and the SILVA small subunit reference database that contains 4,781 high quality verrucomicrobial sequences (Prüsse et al., 2007). The target region for the reverse primer VER_673R (5′ TGC TAC ACC GWG AAT TC 3′) was identified between nucleotide locations 673 and 690, according to the *Escherichia coli* numbering system (Gutell, 1993), taking into consideration the presence of a hairpin followed by a non-canonical pairing of six nucleotides in the 16S rRNA gene secondary structure. When combined with a phylum-specific forward primer VER_37F (5′ TGG CGG CGT GGW TAA GA 3′; Buckley and Schmidt, 2001) the amplified region encompasses the hypervariable regions V1–V4, ensuring accurate taxonomic identification. Primer specificity was further tested against 2,765,278 16S rRNA gene aligned sequences using the Check Probe function of the Ribosomal Database Project II[2] (RDP; Cole et al., 2009) and the Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (NCBI; Altschul et al., 1990). This primer VER_673R yielded a perfect match for 3,211 out of

[1]http://rdp.cme.msu.edu

[2]http://rdp.cme.msu.edu/index.jsp

3,939 high quality verrucomicrobial sequences present in the RDP II, including those belonging to all 133 isolates available throughout the phylum. When searching the database, 35 other sequences were found to be targets for this primer. These sequences, previously identified as *Acidobacteria* (3), *Firmicutes* (4), *Bacteroidetes* (1), *Chloroflexi* (1), *Proteobacteria* (12), and unclassified (14), came from environmental clone libraries and, upon our inspection, could not be classified with at least 80% confidence using the naïve Bayesian rRNA RDP classifier.

The specificity of the *Verrucomicrobia* 16S rRNA gene-targeted primers was experimentally tested with genomic DNA extracted from pure cultures of members of the phylum *Verrucomicrobia* and isolates belonging to the above groups, when available. No PCR amplification was observed for any groups other than *Verrucomicrobia* strains (Supplementary Figure SF1). All isolates yielded positive amplifications with the universal 16S rRNA gene eubacterial primers 8F and 1492R, indicating that the DNA was suitable for amplification.

DNA Extraction and PCR Amplification

Total genomic DNA from soil (0.25 g) was extracted using the Power Soil DNA MoBio DNA Extraction kit (Mobio Laboratories Inc., Carlsbad, CA, USA). Amplification reactions were performed in a final volume of 25 μl containing 10 ng of DNA template, 0.2 μM of each *Verrucomicrobia* 16S rRNA gene specific primer, 2.0 U of Accuprime *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 1X Accuprime PCR buffer II with the following components: 20 mM Tris-HCl (pH 8.4), 200 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 0.5 mM KCl. Amplification was initiated with a 5-min denaturation step at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55 C for 30 s, extension at 68°C for 1 min, then a final extension for 10 min. Aliquots (5 μl) of the PCR products were visualized on ethidium bromide-stained 1% agarose gels.

Clone Library Sequencing

Cloning and transformation were carried out according to the instructions provided with the TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA, USA). *E. coli* transformants were grown on Luria–Bertani medium containing kanamycin (50 μg/ml) and subjected to PCR targeting the cloned insert with primers M13F (5′ GTT GTA AAA CGA CGG CCA GTG 3′) and M13R (5′ CAC ACA GGA AAC AGC TAT G 3′) under the same PCR conditions as above. Amplified products were purified with the kit ExoSAP-IT (USB Corporation, Cleveland, OH, USA) according to manufacturer's instructions and sequenced on an ABI PRISM 3100 automatic sequencer using Big Dye chemistry (Applied Biosystems, Foster City, CA, USA) at the University of Texas Genomics Core Facility (Arlington, TX, USA). A total of 750 sequences were screened and edited in the software Sequencher v.4.2. (Gene Codes Corporation, Ann Arbor, MI, USA). The chimera-check software from the RDP was used to screen sequences for chimeric origin[3]. Taxonomic classification was carried out using the online RDP classifier function.

[3]http://rdp8.cme.msu.edu/cgis/chimera.cgi

Taxonomic and Phylogenetic Diversity Analyses

Trimmed sequences were aligned with the software MUSCLE (Edgar, 2004) and visually inspected before creating a distance matrix with DNAdist software. Operational taxonomic units (OTUs) were established using the furthest neighbor algorithm implemented in the softwared package Mothur (Schloss et al., 2009) with clustering set at 1% of dissimilarity, in accordance to species definition proposed by Stackebrandt and Ebers (2006). OTU richness and Faith's phylogenetic distance (Faith, 1992) were calculated with the software picante (Kembel et al., 2010) in the statistical platform R, and diversity estimators (Shannon and reciprocal Simpson 1/D) were calculated with mothur. Both α and β diversity indices were calculated according to the definition of Whittaker (1972). Taxonomic similarity was calculated by using the Bray–Curtis index, while phylogenetic similarity was calculated by using weighted UniFrac (Lozupone et al., 2007). Patterns of community structure were visualized by non-metric multidimensional scaling (NMDS) of taxonomic (Bray–Curtis) and phylogenetic (weighted UniFrac) similarities. Taxonomic and phylogenetic community compositions were compared among sites by using analyses of similarity (ANOSIM; Clarke, 1993). Class level differences were analyzed with the Library Compare function of the RDP using a confidence threshold of 90%.

For phylogenetic measures of diversity, a phylogenetic tree was constructed with MUSCLE-aligned sequences (Edgar, 2004) and a maximum-likelihood tree was constructed using the gamma + I model with the program PhyML (Guindon et al., 2010). Circular trees were constructed and displayed using the Interactive Tree of Life website[4].

Statistical significances for species richness and diversity indices were calculated with one-way analysis of variance (ANOVA). Fitting of soil variables onto principal coordinate analysis (PCoA) was performed with the vegan package of the software R[5], and the significance of correlation assessed after 999 random permutations.

Quantitative Real Time PCR

Triplicate qPCRs for each soil sample were performed in 20 μL volume containing 1X iTaq fast SYBR Green supermix with ROX as internal reference (Bio-Rad, Hercules, CA, USA), 50 nM of each *Verrucomicrobia* specific primer, and 5 ng of total soil DNA. The ABI 7300 real time PCR system (Applied Biosystems, Calrsbad, CA, USA) was used for quantification. Information about amplification conditions, sensitivity of the quantitative PCR assay for *Verrucomicrobia* specific primers, standard curves, and controls can be found in Supplementary Material.

Nucleotide Sequence Accession Numbers

Partial 16S rRNA gene sequences were deposited in the GenBank database under the accession numbers JF410109 to JF410858.

[4]http://itol.embl.de

[5]http://www.r-project.org

Results

Verrucomicrobia Species Richness

Sampling effort was evaluated through rarefaction curves (Supplementary Figure SF2). The number of OTUs did not reach an asymptote for any of the replicates among the three treatments, but consistently lower numbers of OTUs were observed for the primary forest in comparison with the other two land use treatments. A similar pattern was observed when replicates were pooled within each treatment. Although non-parametric indices are prone to underestimate diversity (Bohannan and Hughes, 2003), they are particularly appropriate for comparative studies such as this one using standardized sample sizes.

Two indices, OTU richness and the Faith's phylogenetic distance, were used to estimate *Verrucomicrobia* diversity. The number of OTUs did not differ significantly among treatments. While 37 OTUs were observed in the primary forest, plots of pasture and secondary forest had 40 and 41 OTUs, respectively (**Figure 1A** – out of 50 sequences). Faith's phylogenetic diversity index was significantly higher for the pasture [$F_{(2,12)} = 3.12$, $P = 0.05$] in comparison to primary and secondary forests, which were similar to one another (**Figure 1B**).

α and β Diversity Analyses

Both non-parametric-derived indices of α diversity, Shannon and the reciprocal of Simpson (1/D), had significantly lower values for the forest than the other two treatments [$F_{(2,12)} = 8.07$, $P < 0.01$; $F_{(2,12)} = 3.53$, $P = 0.05$, respectively] (**Figures 1C,D**). Pasture and secondary forest had similar Shannon indices of 3.33 ± 0.11 and 3.28 ± 0.07, respectively. The reciprocal of the Simpson index was slightly higher for the pasture (22.46 ± 3.93) in comparison to the secondary forest (20.56 ± 1.92).

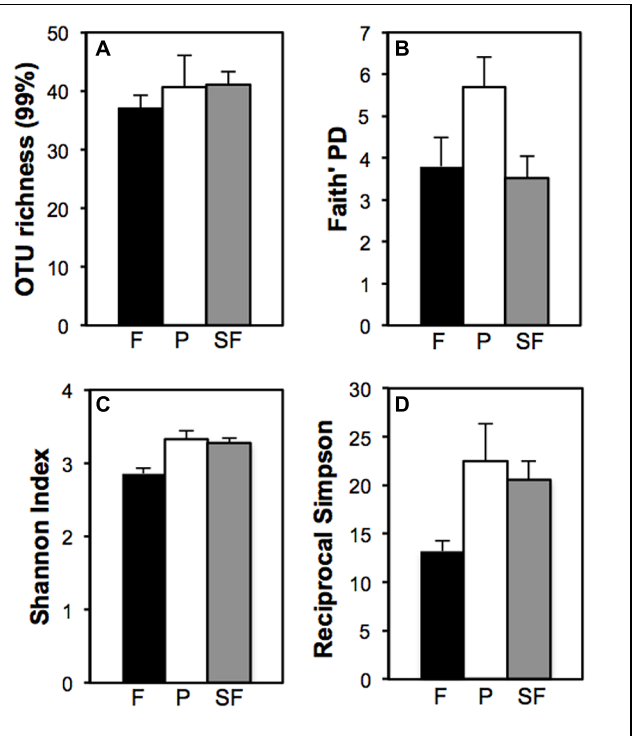

FIGURE 1 | Land use effect on the local diversity of *Verrucomicrobia* in forest (F, black), pasture (P, white), secondary forest (SF, gray): (A) total species richness, (B) Faith's phylogenetic diversity, (C) Shannon Index, and (D) Reciprocal Simpson Index (1/D). Bars represent SEs.

The *Verrucomicrobia* community composition from pasture soils was significantly different from that observed in primary and secondary forests. Analyses of similarity using both taxonomic ($R = 0.501$, $P < 0.001$) and phylogenetic ($R = 0.476$, $P < 0.001$) measures revealed a distinct separation between pasture and forest samples (**Figures 2A,B**). Two measures of β diversity were used to estimate the similarity in community structure among soil samples. Both UniFrac (phylogenetic) and Bray–Curtis (taxonomic) coefficients decreased with forest-to-pasture conversion, a trend which was reversed for both indices with pasture abandonment and re-establishment of secondary forest [$F_{(2,57)} = 5.86$, $P < 0.001$; $F_{(2,12)} = 32.2$, $P < 0.001$, respectively, Supplementary Figure SF3].

Phylogenetic Novelty

All 750 sequences were identified using the naïve Bayesian rRNA RDP classifier based on 99% sequence identity (**Figure 3**). A large majority of pasture *Verrucomicrobia* sequences grouped into clades different from those obtained from forest soils. *Verrucomicrobia* sequences cloned from the secondary forest samples were evenly distributed throughout the phylogenetic tree. A total of 302 sequences (40.3%) retrieved in this study were not previously observed in any other study. The majority of the novel sequences ($n = 124$) originated from the forest samples, followed by the secondary forest ($n = 113$) and the pasture ($n = 65$).

The majority of the sequences (90%) belonged to two classes, *Spartobacteria* and subphylum 3. The relative proportion of sequences classified as subphylum 3 in the pasture (26.8%) was significantly higher ($P < 0.01$) than the number of subphylum 3 sequences found in the forest (15.2%, **Figure 4**). There was an equivalent decrease of 11.6% in the number of *Spartobacteria* 16S rRNA gene sequences recovered from pasture samples. The relative proportions of *Verrucomicrobia* sequences in the primary and secondary forests were similar for both the *Spartobacteria* and subphylum 3 with a minor variation of 1.2 and 4%, respectively.

Influence of Soil Factors on the *Verrucomicrobia* Community

To explore potential drivers of the community, we performed environmental fitting of 22 different soil variables onto the ordination plot via Principle Coordinate Analysis (PCoA, Supplementary Figure SF4). Five variables were linked to *Verrucomicrobia* phylogenetic community structure, with total C yielding the most significant results ($P < 0.05$). Increases in organic matter, total N, and pH were linked to 16S rRNA gene sequences obtained from pasture, while an increase in potential acidity (H + Al) was linked to those from forest samples.

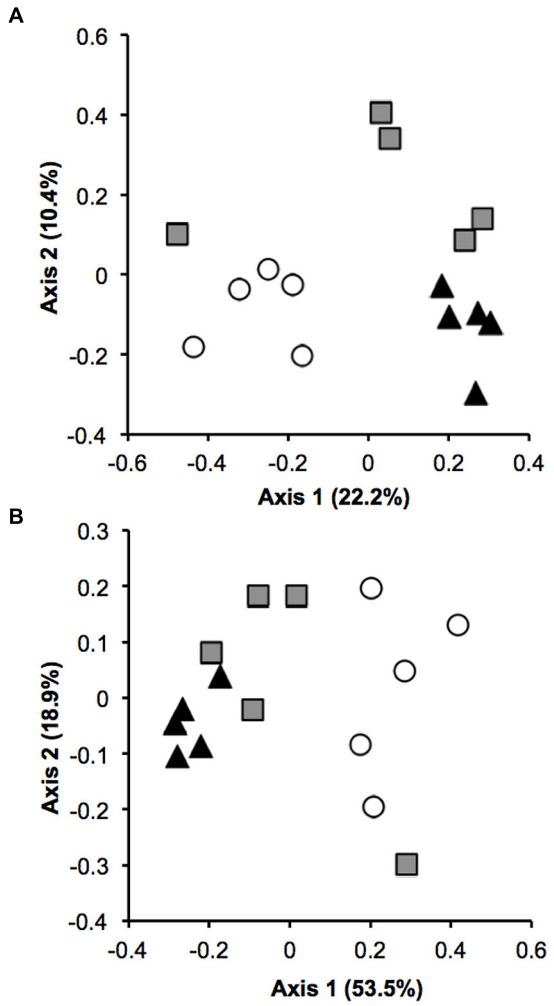

FIGURE 2 | *Verrucomicrobia* community composition of forest (triangle), pasture (circle), and secondary forest (square). Non-metric multidimensional scaling (NMDS) plot of **(A)** taxonomic similarity (Bray–Curtis) and **(B)** phylogenetic similarity (weighted UniFrac).

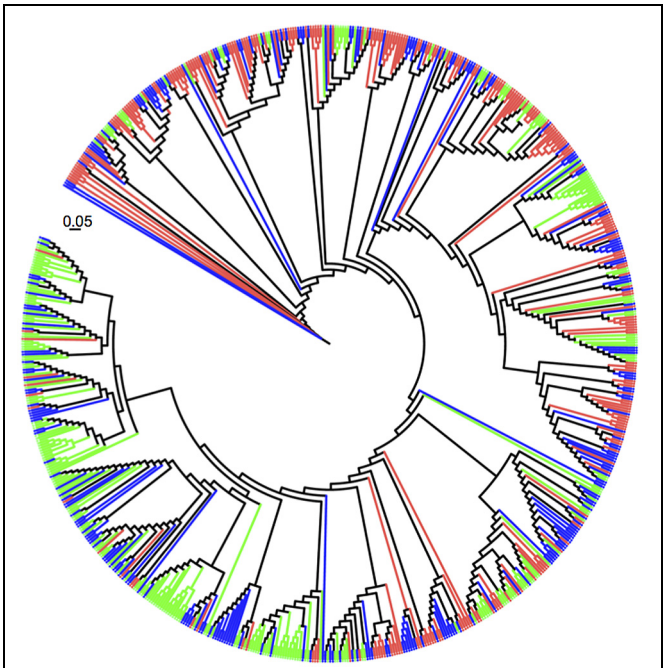

FIGURE 3 | Unrooted maximum likelihood tree of 750 sequences belonging to *Verrucomicrobia* 16S rRNA genes recovered from soils: Amazon forest (green), pasture (red), and secondary forest (blue). The scale bar represents the number of substitutions per nucleotide position.

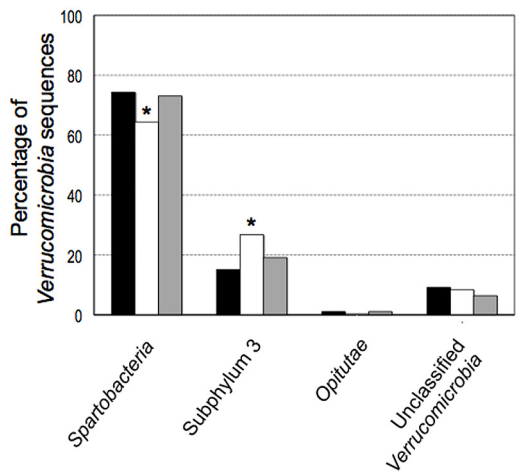

FIGURE 4 | Phylogenetic comparison among classes of the phylum *Verrucomicrobia* 16S rRNA gene sequences from different land uses in the Amazon: forest (black), pasture (white) and secondary forest (gray). Symbol * indicate significant differences at $P = 0.01$.

Abundance of *Verrucomicrobia*

The detection limit was established to be approximately 10^3 copies of the target sequence per gram of soil with linear standard curves over six orders of magnitude. Gene copy numbers were consistently higher ($P < 0.05$) for pasture samples (1.9×10^7 copies per gram of soil), followed by secondary forest (9.1×10^6) and primary forest soil samples (2.4×10^6).

Discussion

Soils are known to have higher taxonomic and functional diversity than any other environment (Whitman et al., 1998). Earlier estimates suggested that 3.8×10^3 to 10^7 microbial species are present in a gram of soil (Curtis et al., 2002; Gans et al., 2005) and 32 phyla have already been detected (Janssen, 2006). We reason that if one is interested in understanding how soil microbial communities respond to environmental disturbances, focusing on particular groups is crucial, since not all groups are likely to respond in the same manner.

To capture the diversity of the phylum *Verrucomicrobia*, we designed a new 16S rRNA gene PCR primer (VER_37F) and paired it with a previously tested primer VER_673R (Buckley and

Schmidt, 2001). This new primer pair differs from *Eubacterial* primers (515F/806R) proposed by Bergmann et al. (2011). While the primers 515F/806R are relatively unbiased against the *Verrucomicrobia*, they are not specific for this phylum. The primer pair VER_37F-VER_673R was highly specific for the target phylum when tested *in silico* and experimentally with the recovery of 750 *Verrucomicrobia* sequences from Amazon soils. Although our sample is not as comprehensive as one obtained from a high-throughput sequencing study, the longer reads we generated provide more detailed taxonomic assignments for lower rank hierarchies (Wang et al., 2007) of the numerically dominant OTUs present in our soil samples than would be possible with a high-throughput study. Both methods use PCR based information and large-scale patterns observed in soils with the PCR dependent method T-RFLP (Fierer and Jackson, 2006) were unchanged when the same samples were subjected to pyrosequencing (Lauber et al., 2009).

Our results indicate the presence of a diverse *Verrucomicrobia* community that was not previously observed in tropical rainforests through the use of oligonucleotides targeting the domain *Eubacteria*. An important outcome of our targeted approach is the high number of novel sequences observed. Using an OTU definition based on 99% sequence identity, 40.2% of the *Verrucomicrobia* sequences were novel, suggesting that the microbial diversity of tropical ecosystems remains largely unknown. More importantly, this high diversity in Amazon soils is impacted by forest-to-pasture conversion. Both non-parametric indices (Shannon and the reciprocal of Simpson) showed increased values of community diversity for soils collected from pasture. These findings are in agreement with previous studies, in which forest-to-pasture conversions resulted in increased α diversity of bacterial communities (Jesus et al., 2009; Rodrigues et al., 2013). We also documented an increase in β diversity (Anderson et al., 2011) as measured by a decrease in taxonomic (Bray–Curtis) and phylogenetic (UniFrac) similarities. The response of *Verrucomicrobial* beta diversity contrasts the response of the *Bacteria* as a whole, which showed increased taxonomic homogenization following pasture conversion. This further demonstrates the utility of focused analysis on particular microbial groups, which can often differ from patterns observed within the larger community.

It is possible that the differences we observed across land type could be the result of spatial variability in soil conditions that were present in the three sites before land use change occurred. This is unlikely, for two reasons. First, all of the study sites have identical soil type, drainage, slope, and are geographically located close to each other (Cenciani et al., 2009). Second, this chronosequence of land uses has been systematically studied over the past 25 years and spatial variability was found to be small in comparison to land use history (Moraes et al., 1995; Steudler et al., 1996; Neill et al., 1997).

We observed that the community composition of *Verrucomicrobia* was altered with land use change in two distinct ways. First, the relative proportions of taxa varied with land type. There is a clear distinction between clades formed by OTUs observed in the forest and those found in the pasture (**Figure 3**, note that forest sequences marked in green are mostly at the bottom of the circular tree while pasture sequences, in red, are positioned at the top). This alteration can be attributed to the increase in 16S rRNA gene sequences belonging to the subphylum 3 and a concurrent decrease in the number of *Spartobacteria* sequences. Out of the seven taxonomic classes within the *Verrucomicrobia* (Hedlund, 2011), these two groups comprise the majority of the 16S rRNA gene sequences and isolates retrieved from soil (Joseph et al., 2003; Sangwan et al., 2004; Janssen, 2006). We cannot characterize the increase in the relative proportion of subphylum 3 sequences as a shift in dominance, as the *Spartobacteria* sequences remain above 60% of the total in all sites. However, our results are consistent with habitat filtering, the process by which habitat characteristics exert selection pressure on community composition (Webb et al., 2002). We documented that at least 11.6% of the verrucomicrobial community was altered as a consequence of conversion. It is currently unknown if these forest inhabitants carry unique traits. Genomes of the first isolates of both *Spartobacteria* and subphylum 3 have been sequenced (Kant et al., 2011a,b), and a comparative analysis may provide insights into their ecophysiological differences and reasons for habitat selection. Secondly, the abundance of *Verrucomicrobia* increased with conversion of forest to pasture. We hypothesize that this response is a direct result of rich exudates released by the large root system of C4 plants, such as grasses (Feigl et al., 1995; Haichar et al., 2014). Two lines of evidence support this is hypothesis: (A) higher abundances members of the *Verrucomicrobia* have been observed in the plant rhizosphere in comparison to bulk soil (Idris et al., 2004; Kielak et al., 2008; Jesus et al., 2010; Rocha et al., 2010); and (B) attempts to isolate members of *Spartobacteria* from soil have only been successful when plant polymers or sugars were added to the medium (Sangwan et al., 2004). Taken together, the above results suggest that plants may be an important driving force,

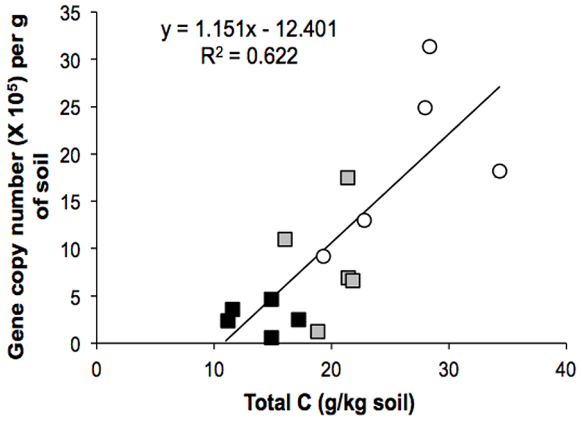

FIGURE 5 | Regression analysis between the abundance of *Verrucomicrobia* 16S rRNA genes and total carbon in the Amazon soils. The correlation coefficient (*r*) between the two variables is 0.80 ($P = 0.0016$). Symbols: forest (black square), pasture (open circle), secondary forest (gray square).

albeit difficult to quantify, on the community composition of *Verrucomicrobia* in soils.

Given the genetic and ecological consequences of tropical biodiversity loss, we asked whether there are signs of resilience within the forest *Verrucomicrobia* community (Allison and Martiny, 2008). Our results indicate that restoration of community composition is under way after 7 years of disturbance (pasture from 1987 to 1994), followed by 10 years of reestablishment of a secondary forest (from 1994 to 2004). After many years of cessation of the disturbance, the above ground community has not returned to its original species composition (Feigl et al., 2006), nor have many of the soil attributes been restored to their original values (Cenciani et al., 2009). Pairwise comparisons of the secondary forest samples revealed that the *Verrucomicrobia* community structures are similar to those observed for the primary forest. Furthermore, *Verrucomicrobia* communities of primary and secondary forests respond to the same environmental variables (Supplementary Figure SF4). Because phylogenetic relationships are not always predictors of microbial physiology, it remains to be determined whether similar phylogenetic taxa perform similar functional roles in the restored ecosystem. Nonetheless, our results are encouraging from the standpoint of community conservation and restoration ecology. Approximately 50% of abandoned pastures in the Amazon rainforest are estimated to be in a secondary forest succession (Davidson et al., 2012). Young tropical forests are increasing in importance for genetic diversity conservation and stability of degraded environments, but their microbial communities, nutrient cycling processes, and ecosystem services remain understudied. Our study, although limited, suggests that membership recovery may be possible.

Environmental biotic and abiotic characteristics are important determinants of the ecological niche occupied by a particular population and we sought to correlate the presence of members of the *Verrucomicrobia* with 22 soil variables. While potential acidity (H + Al) was the only variable linked to the *Verrucomicrobia* community from the primary forest, increases in organic matter, pH, total *N*, and total *C* were significantly associated with those from the pasture. These soil properties are long lasting effects of the slash and burn procedure (Neill et al., 1997) and drastic alteration of plant species composition to actively growing grasses, with high deposition of *C* to soil (Feigl et al., 1995). We examined the influence of *C* content on the abundance of *Verrucomicrobia* and observed a strong correlation ($r = 0.80$, $P = 0.0016$; **Figure 5**), indicating that *C* may be an important factor in determining the structure of this particular microbial group. Carbon resource heterogeneity has been suggested as a driving force for microbial community structure (Zhou et al., 2002) and it is likely that changes in the above ground community will alter carbon content and availability of different carbon-containing compounds. Our results suggest that the *Verrucomicrobia* community increases its abundance in soil in response to *C*, in agreement with previous metagenomic studies that found that the distribution of carbohydrate metabolism genes from *Verrucomicrobia* were associated with shifts in *C* dynamics (Fierer et al., 2013).

Conclusion

Although land use change has profound consequences for plant and animal biodiversity, its effect on microorganisms is not well understood. By targeting the phylum *Verrucomicrobia* at class level of taxonomic and phylogenetic resolution, we documented changes in the relative abundance of specific groups with forest-to-pasture conversion, as well as an increase in both alpha and beta diversity. We also identified carbon content as an important environmental factor associated with soil *Verrucomicrobia* abundance. By solely focusing on the *Verrucomicrobia*, we were able to (1) increase the number of novel *Verrucomicrobia* 16S rRNA gene sequences detected, (2) characterize changes in taxonomic and phylogenetic diversity at the class level, and (3) make inferences about the environmental requirements for yet-to-be cultured members of this phylum. Although we documented strong shifts in response to deforestation and pasture establishment, 10 years after disturbance, the *Verrucomicrobia* community shows signs of recovery to its original composition, suggesting that this group shows resilience to ecosystem modifications.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00779

References

Allison, S. D., and Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11512–11519. doi: 10.1073/pnas.0801925105

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anderson, M. J., Crist, T. O., Chase, J. M., Vellend, M., Inouye, B. D., Freestone, A. L., et al. (2011). Navigating the multiple meanings of ß diversity: a

roadmap for the practicing ecologist. *Ecol. Lett.* 14, 19–28. doi: 10.1111/j.1461-0248.2010.01552.x

Bastos, T., and Diniz, T. D. de A. S. (1982). Avaliação de clima do estado de rondônia para desenvolvimento agrícola. *Boletim Pesquisa* 44, 28.

Bergmann, G. T., Bates, S. T., Eilers, K. G., Lauber, C. L., Caporaso, J. G., Walters, W. A., et al. (2011). The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biol. Biochem.* 43, 1450–1455. doi: 10.1016/j.soilbio.2011.03.012

Bohannan, B. J. M., and Hughes, J. (2003). New approaches for analyzing biodiversity data. *Curr. Opin. Microbiol.* 6, 282–287. doi: 10.1016/S1369-5274(03)00055-9

Buckley, D. H., and Schmidt, T. M. (2001). Environmental factors influencing the distribution of rRNA from Verrucomicrobia in soil. *FEMS Microbiol. Ecol.* 35, 105–112. doi: 10.1111/j.1574-6941.2001.tb00793.x

Cenciani, K., Lambais, M. R., Cerri, C. C., Azevedo, L. C. B., and Feigl, B. J. (2009). Bacteria diversity and microbial biomass in forest, pasture and fallow soils in the Southwestern Amazon Basin. *Rev. Brasil. Cien. Solo* 33, 907–916. doi: 10.1590/S0100-06832009000400015

Clarke, K. R. (1993). Non-parametric multivariate analysis of changes in community structures. *Aust. J. Ecol.* 18, 117–143. doi: 10.1111/j.1442-9993.1993.tb00438.x

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

Curtis, T. P., Sloam, W. T., and Scannell, J. W. (2002). Estimating prokaryotic diversity and its limits. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10494–10499. doi: 10.1073/pnas.142680199

Da Silva, J. M. C., Rylands, A. B., and Fonseca, G. A. B. (2005). The fate of the Amazonian areas of endemism. *Conserv. Biol.* 19, 689–694. doi: 10.1111/j.1523-1739.2005.00705.x

Davidson, E. A., Araújo, A. C., Artaxo, P., Balch, J. K., Brown, E. F., Bustamante, M. M. C., et al. (2012). The Amazon basin in transition. *Nature* 481, 321–328. doi: 10.1038/nature10717

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Feigl, B. J., Melillo, J., and Cerri, C. C. (1995). Changes in the origin and quality of soil organic matter after pasture introduction in Rondonia (Brazil). *Plant Soil* 175, 21–29. doi: 10.1007/BF02413007

Feigl, B. J., Cerri, C., Piccolo, M., Noronha, N., Augusti, K., Melillo, J., et al. (2006). Biological survey of a low-productivity pasture in Rondonia state, Brazil. *Outlook Agricul.* 35, 199–208. doi: 10.5367/000000006778536738

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.*103, 626–631. doi: 10.1073/pnas.0507535103

Fierer, N., Ladau, J., Clemente, J. C., Leff, J. W., Owens, S. M., Pollard, K. S., et al. (2013). Reconstructing the microbial diversity and function of pre-agricultural tallgrass prairie soils in the United States. *Science* 342, 621–624. doi: 10.1126/science.1243768

Gans, J., Woilinsky, M., and Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309, 1387–1390. doi: 10.1126/science.1112665

Gibson, L., Lee, T. M., Koh, L. P., Brook, B. W., Gardner, T. A., Barlow, J., et al. (2011). Primary forests are irreplaceable for sustaining tropical biodiversity. *Nature* 478, 378–381. doi: 10.1038/nature10425

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Gutell, R. R. (1993). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Res.* 21, 3051–3055. doi: 10.1093/nar/21.13.3051

Haichar, F. el Z., Santaella, C., Heulin, T., and Achouak, W. (2014). Root exudates mediated interactions belowground. *Soil Biol. Biochem.* 77, 69–80. doi: 10.1016/j.soilbio.2014.06.017

Hedlund, B. P. (2011). "Phylum XXIII. Verrucomicrobia phyl. Nov," in *Bergey's Manual of Systematic Bacteriology*, eds N. R. Krieg, W. Ludwig, W. R. Whitman, B. P. Hedlund, B. J. Paster, J. T. Staley, et al., (New York: Williams & Wilkins), 795–841.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W. W., and Sessitsch, A. (2004). Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl. Environ. Microbiol.* 70, 2667–2677. doi: 10.1128/AEM.70.5.2667-2677.2004

Janssen, P. H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719–1729. doi: 10.1128/AEM.72.3.1719-1728.2006

Jesus, E. C., Marsh, T. L., Tiedje, J. M., and Moreira, F. M. S. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Jesus, E. C., Susilawati, E., Smith, S. L., Wang, Q., Chai, B., Farris, R., et al. (2010). Bacterial communities in the rhizosphere of biofuel crops grown on marginal lands as evaluated by 16S rRNA gene pyrosequences. *Bioenerg. Res.* 3, 20–27. doi: 10.1007/s12155-009-9073-7

Joseph, S. J., Hugenholtz, P., Sangwan, P., Osborne, C. A., and Janssen, P. H. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl. Environ. Microbiol.* 69, 7210–7215. doi: 10.1128/AEM.69.12.7210-7215.2003

Kant, R., van Passel, M. W. J., Sangwan, P., Palva, A., Lucas, S., Copeland, A., et al. (2011a). Genome sequence of *Pedosphaera parvula* Ellin514, an aerobic verrucomicrobial isolate from pasture soil. *J. Bacteriol.* 193, 2900–2901. doi: 10.1128/JB.00299-11

Kant, R., van Passel, M. W. J., Palva, A., Lucas, S., Copeland, A., Lapidus, A., et al. (2011b). Genome sequence of *Chthoniobacter flavus* Ellin428, an aerobic heterotrophic soil bacterium. *J. Bacteriol.* 193, 2902–2903. doi: 10.1128/JB.00295-11

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: r tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kielak, A., Pijl, A. S., van Veen, J. A., and Kowalchuk, G. A. (2008). Differences in vegetation composition and plant species identity lead to only minor changes in soil-borne microbial communities in a former arable field. *FEMS Microbiol. Ecol.* 63, 372–382. doi: 10.1111/j.1574-6941.2007.00428.x

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi: 10.1128/AEM.00335-09

Laurance, W. F., Cochrane, M. A., Bergen, S., Fearnside, P. M., Delamonica, P., Barber, C., et al. (2001). The future of the Brazilian Amazon. *Science* 291, 438–439. doi: 10.1126/science.291.5503.438

Lewinsohn T. M., and Prado, P. I. (2005). How many species are there in Brazil? *Conserv. Biol.* 19, 619–626. doi: 10.1111/j.1523-1739.2005.00680.x

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Martinez-Garcia, M., Brazel, D. M., Swan, B. K., Arnosti, C., Chain, P. S. G., Reitenga, K. G., et al. (2012). Capturing single cell genomes of active polysaccharide degraders: an unexpected contribution of *Verrucomicrobia*. *PLoS ONE* 7:35314. doi: 10.1371/journal.pone.0035314

Montecchia, M. S., Tosi, M., Soria, M. A., Vogrig, J. A., Sydorenko, O., and Correa, O. S. (2015). Pyrosequencing reveals changes in soil bacterial communities after conversion of Yungas forest to agriculture. *PLoS ONE* 10:e0119426. doi: 10.1371/journal.pone.0119426

Moraes, J. F. L., Cerri, C. C., Melillo, J. M., Kicklighter, D., Neill, C., Steudler, P., et al. (1995). Soil carbon stocks of the Brazilian Amazon basin. *Soil Sci. Soc. Am. J.* 59, 244–247. doi: 10.2136/sssaj1995.03615995005900010038x

Neill, C., Piccolo, M. C., Cerri, C. C., Steudler, P. A., Melillo, J. M., and Brito, M. M. P. (1997). Net nitrogen mineralization and net nitrification rates in soils following deforestation for pasture across the southwestern Brazilian Amazon basin landscape. *Oecologia* 110, 243–252. doi: 10.1007/s004420050157

Prober, S. M., Leff, J. W., Bates, S. T., Borer, E. T., Firn, J., Harpole, W. S., et al. (2015). Plant diversity predicts beta but not alpha diversity of soil

microbes across grasslands worldwide. *Ecol. Lett.* 18, 85–95. doi: 10.1111/ele.12381

Prüsse, E., Quast, C., Knittel, K., Fuchs, B., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Rocha, U. N., da van Elsas, J. D., and van Overbeek, L. S. (2010). Real-time PCR detection of Holophagae (Acidobacteria) and Verrucomicrobia subdivision 1 groups in bulk and leek (Allium porrum) rhizosphere soils. *J. Microbiol. Methods* 83, 141–148. doi: 10.1016/j.mimet.2010.08.003

Rodrigues, J. L. M., Pellizari, V., Mueller, R., Baek, K., Jesus, E. C., Paula, F. S., et al. (2013). Conversion of the Amazon Rainforest to agriculture results in biotic homogenization of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 110, 988–993. doi: 10.1073/pnas.1220608110

Sala, O. E., Chapin, F. S. III, Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., et al. (2000). Global biodiversity scenarios for the year 2100. *Science* 287, 1770–1774. doi: 10.1126/science.287.5459.1770

Sangwan, P., Chen, X., Hugenholtz, P., and Janssen, P. H. (2004). Chthoniobacter flavus gen. nov., sp. nov., the first pure-culture representative of subdivision two, Spartobacteria classis nov., of the phylum Verrucomicrobia. *Appl. Environ. Microbiol.* 70, 5875–5881. doi: 10.1128/AEM.70.10.5875-5881.2004

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Soares-Filho, B. S., Nepstad, D. C., Curran, L. M., Cerqueira, G. C., Garcia, R. A., Ramos, C. A., et al. (2006). Modelling conservation in the Amazon basin. *Nature* 440, 520–523. doi: 10.1038/nature04389

Stackebrandt, E., and Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol. Today* 11, 6–9.

Steudler, P. A., Melillo, J. M., Feigl, B. J., Neill, C., Picollo, M., and Cerri, C. C. (1996). Consequence of forest-to-pasture conversion on CH4 fluxes in the Brazilian Amazon Basin. *J. Geophys. Res.* 101, 18547–18554. doi: 10.1029/96JD01551

Suleiman, A. K. A., Manoeli, L., Boldo, J. T., Pereira, M. G., and Roesch, L. F. W. (2013). Shifts in soil bacterial community after eight years for land-use change. *Syst. Appl. Microbiol.* 36, 137–144. doi: 10.1016/j.syapm.2012.10.007

Wang, Q., Gerrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wearn, O. R., Reuman, D. C., and Ewers, R. M. (2012). Extinction debt and windows of conservation opportunity in the Brazilian Amazon. *Science* 337, 228–232. doi: 10.1126/science.1219013

Webb, C. O., Ackerly, D. D., McPeek, M. A., and Donoghue, M. J. (2002). Phylogenies and community ecology. *Ann. Rev. Ecol. Evol. Syst.* 33, 475–505. doi: 10.1146/annurev.ecolsys.33.010802.150448

Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6578–6583. doi: 10.1073/pnas.95.12.6578

Whittaker, R. H. (1972). Evolution and measure of species diversity. *Taxon* 21, 213–251. doi: 10.2307/1218190

Zhou, Z., Xia, B., Treves, D. S., Wu, L.-Y., Marsh, T. L., O'Neill, R. V., et al. (2002). Spatial and resource factors influencing high microbial diversity in soil. *Appl. Environ. Microbiol.* 68, 326–334. doi: 10.1128/AEM.68.1.326-334.2002

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Tree Plantation Systems Influence Nitrogen Retention and the Abundance of Nitrogen Functional Genes in the Solomon Islands

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Tree mono-plantations are susceptible to soil nutrient impoverishment and mixed species plantations have been proposed as a way of maintaining soil fertility while enhancing biodiversity. In the Solomon Islands, mixed species plantations where teak (*Tectona grandis*) is inter-planted with a local tree species (*Flueggea flexuosa*) have been used as an alternative to teak mono-plantations and are expected to increase soil microbial diversity and modify microbial biogeochemical processes. In this study, we quantified the abundance of microbial functional genes involved in the nitrogen (N) cycle from soil samples collected in teak, flueggea, and mixed species plantations. Furthermore, we measured soil properties such as pH, total carbon (C) and total N, stable N isotope composition ($\delta^{15}N$), and inorganic N pools. Soil pH and $\delta^{15}N$ were higher under teak than under flueggea, which indicates that intercropping teak with flueggea may decrease bacterial activities and potential N losses. Higher C:N ratios were found under mixed species plantations than those under teak, suggesting an enhancement of N immobilization that would help preventing fast N losses. However, inorganic N pools remained unaffected by plant cover. Inter-planting teak with flueggea in mixed species plantations generally increased the relative abundance of denitrification genes and promoted the enrichment of *nosZ*-harboring denitrifiers. However, it reduced the abundance of bacterial *amoA* (ammonia monooxygenase) genes compared to teak mono-plantations. The abundance of most denitrification genes correlated with soil total N and C:N ratio, while bacterial and archeal nitrification genes correlated positively with soil NH_4^+ concentrations. Altogether, these results show that the abundance of bacterial N-cycling functional guilds vary under teak and under mixed species plantations, and that inter-planting teak with flueggea may potentially alleviate N losses associated with nitrification and denitrification and favor N retention. Mixed plantations could also allow an increase in soil C and N stocks without losing the source of income that teak trees represent for local communities.

Keywords: *Tectona grandis*, *Flueggea flexuosa*, mixed species plantations, $\delta^{15}N$, tropical soil, qPCR

INTRODUCTION

In tropical countries such as the Solomon Islands where deforestation rates are high, tree plantations are seen as a way to counteract soil degradation by restoring vegetation cover while decreasing the existing pressure on native forests (Wolfe et al., 2015). However, most established tree plantations are mono-plantations that can cause the same environmental problems as other monoculture systems, namely higher pest or disease occurrence and a modification of biogeochemical cycles and nutrient availability (Rachid et al., 2013). In order to maintain soil fertility and to enhance biodiversity, mixed plantations are being promoted and are expected to reduce plant competition for nutrients and increase soil carbon (C) and nitrogen (N) pools (Montagnini, 2000; Balieiro et al., 2008; Vigulu, 2015). Mixed-species systems are also expected to enhance soil microbial diversity by increasing the variety of carbon (C) based resources and the heterogeneity of spatial patterns in soil properties (Thevathasan and Gordon, 2004).

Many essential soil processes are primarily mediated by microbial communities (Bissett et al., 2013). Land management practices and changes in plant species composition are known to impact microbially driven processes in soils through the alteration of bacterial communities that will thereby modify nutrient availability to plants or nutrient losses from the ecosystem (Chen et al., 2003; Kourtev et al., 2003; Lindsay et al., 2010). Soil microorganisms play a central role in organic matter decomposition and in the cycling of major plant nutrients, including N (Hallin et al., 2009; Bissett et al., 2011). Yet, the importance of soil microbial diversity is often overlooked when establishing forest plantations (Levy-Booth and Winder, 2010). With the growing concerns about intensive mono-plantations comes an increasing interest in the management of soil fertility and soil bacterial communities to enhance tree growth and productivity (Lacombe et al., 2009). However, how the establishment of mixed plantations influences the functions of soil bacterial communities, as measured through microbial functional genes (MFGs), and how this relates to nutrient cycling remains to be understood.

In the Solomon Islands, teak (*Tectona grandis* L.f.) is often grown in smallholder plantations in order to rehabilitate the logged-over rainforests while providing a source of income to landowners (Vigulu, 2015). Teak is an economically important timber tree species grown in tropical and sub-tropical countries for its highly durable hardwood (Miranda et al., 2011). Mostly cultivated in monoculture plantations in 20–40 years rotation, its height can reach more than 20 m at maturity (Ladrach, 2009). Teak grows well on a broad range of soils but its growth has been reported to be optimal on deep and well-drained sandstones, with neutral or acid pH and high calcium, phosphorus, potassium, N, and organic matter contents (Kadambi, 1972). Currently, a new plantation system is being introduced in the Solomon Islands where teak is intercropped with a local tree species (*Flueggea flexuosa* Muell. Arg.) in order to overcome the reluctance of growers to thin pure teak stands. Flueggea, a small to medium tree typically 10–16 m tall, is traditionally used for house building and fencing in the Solomon Islands (Thomson, 2006). Flueggea was considered as a good candidate species for intercropping with teak as roots from both trees seem to occupy different soil depth. While teak has extensive horizontal and vertical roots and occupy a large portion of the soil volume, flueggea's root system usually develops laterally, near the soil surface (Thomson, 2006; Vigulu, 2015).

The implementation of mixed-species systems is likely to influence nutrient cycling and the abundance of MFG associated with nutrient cycling (Rachid et al., 2013). Therefore, we aimed to determine the abundance of MFG involved in N cycling under teak mono-plantations, flueggea mono-plantations and mixed-species systems and evaluate differences in soil N pools due to tree cover. To accomplish this, we assessed the abundances of genes involved in nitrification (bacterial and archeal *amoA*), nitrogen fixation (*nifH*), and denitrification (*narG*, *nirS*, *nirK*, and *nosZ*) as indicators of microbial trait abundances (Wieder et al., 2013), and measured soil variables such as pH, total C and N content, NO_3^-, and NH_4^+. Soil N isotope composition ($\delta^{15}N$) was also analyzed as an indicator of N cycling rates (Högberg, 1997; Hietz et al., 2011; Reverchon et al., 2014). Finally, we discussed the existing relationships between MFG and soil characteristics in tree plantation soils.

MATERIALS AND METHODS

Sampling Design

The study site is located at Poitete, on Kolombangara Island, in the Western Province of the Solomon Islands (8° 05′ 16.33″ S and 157° 08′ 46.62″ E; **Figure 1**). The average annual temperature in the area is 28°C and the average rainfall is 3,600 mm, relatively evenly distributed throughout the year (**Supplementary Figure S1**). The soil at the study site is an Oxisol (Vigulu, 2015).

Tree plantations were established in 2009 on land formerly covered with regenerated secondary tropical forests. Plantations consisted of mono-plantations of teak, mono-plantations of flueggea and mixed-species plantations (consecutive rows of teak and flueggea). All plantations were adjacent and therefore established on the same soil type, and had a planting density of 833 stems per hectare (spacing 4 m × 3 m). Three years after planting, teak trees were 16 m high with a diameter at breast height (dbh) of 19 cm, while flueggea trees were 12 m high with a dbh of 13 cm (Vigulu, 2015). The crowns and root systems of both trees were well-developed, with crown radius and root growth of teak trees being larger than those of flueggea (Vigulu, 2015).

Three replicated plots of 24 m × 24 m were established per plantation and a 24-m transect was drawn in the middle of each plot (nine transects in total). In the mixed-species plots, transects were drawn in order to cross alternatively rows of teak and rows of flueggea. Five sampling points were established 4 m apart along each transect. At each sampling point, soil samples were collected on the transect and 4 m perpendicularly on each side of the transect (three samples per sampling point), mixed, and bulked to constitute one composite sample per sampling point. All samples were collected from 0 to 15 cm soil layer, with a shovel. Soil samples were then immediately sieved (2 mm) and refrigerated

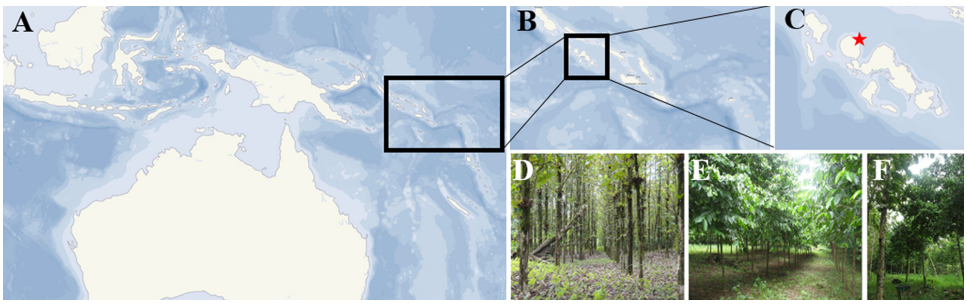

FIGURE 1 | Smallholder tree plantation systems in Solomon Islands. The field site is located within the Pacific region **(A)**, in Solomon Islands **(B)**, at Kolombangara, Western Province **(C)**, where teak **(D)**, flueggea **(E)**, and mixed-species **(F)** plantations were established in 2009.

until analysis (maximum of 5 days). The total number of samples per sampling was 45. Samplings were carried out in December 2012, May 2013, and December 2013.

Soil Chemical Analyses

Total C, total N, and $\delta^{15}N$ of soil samples were determined by mass spectrometry (spectrometer GV Isoprime, Manchester, UK), following the procedure described in He et al. (2008). Soil pH was measured in water (1:5 ratio). Soil NH_4^+ and NO_3^- concentrations were determined by KCl extraction using a SmartChem 200 Discrete Chemistry Analyser as described in Bai et al. (2012). All soil NO_3^- concentrations were below detection level and were thus not included in the subsequent statistical analyses.

Quantification of the N-Cycle-Associated MFG

Soil DNA extractions were carried out within 1 week after sampling. DNA was extracted from 0.3 g of each soil sample using the MoBio Powersoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions, with a final elution in deionised water. The quantity and quality of the extracted DNA were verified with a Nanodrop spectrophometer (Thermoscientific) and diluted in deionised water (1:10).

The total bacterial community was quantified by quantitative PCR (qPCR) using the V3 hypervariable region of the 16S rRNA gene as a molecular marker. The abundances of functional genes *narG*, *nirS*, *nirK*, *nosZ*, *nifH*, and bacterial and archeal *amoA* (AOB and AOA respectively) were quantified using the primers and thermal cycling conditions described in Supplementary Table S1. Reactions were carried out in an Eppendorf Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) in duplicate. Quantification was based on the fluorescence intensity of the SYBR Green dye (Takara) during amplification. Standard curves were obtained using 10-fold serial dilutions of plasmid DNA containing cloned *narG*, *nirS*, *nirK*, *nosZ*, *nifH*, *amoA* and 16S rRNA genes and spanning seven orders of magnitude. The 20 μL PCR mixture contained 10 μL of SYBR green PCR Master Mix [Takara SYBR Premix Ex Taq (Perfect Real Time)], 0.4 μL of each primer (10 μM) and approximately 8 ng DNA. Melting curves and agarose gels of PCR products were used at the end of each qPCR to check amplification specificity and purity of negative controls. Negative controls gave null or negligible values, and PCR efficiency for the different assays ranged from 90 to 99%. The presence of PCR inhibitors in DNA extracted from soil was estimated by a 1:10 soil DNA dilution; no inhibition was detected. All qPCR reactions were carried out immediately after DNA extraction.

The measured cycle threshold (Ct) values of standards quantification were calibrated by placing the threshold lines at the same level for each gene, to account for the different times at which samples from December 2012, May 2013, and December 2013 were processed for qPCR. Gene data were expressed in number of gene copies ng^{-1} DNA rather than per gram of soil to minimize any bias related to soil DNA extraction efficiency (Čuhel et al., 2010; Correa-Galeote et al., 2013; Rachid et al., 2013).

Statistical Analyses

A repeated measures two-way analysis of variance (ANOVA) followed by Tukey HSD tests were conducted to detect the effects of plantation type and of sampling time on the measured soil variables and on the abundance of MFG. All data were tested for normality using Shapiro-Wilk normality test and for homogeneity of variance with Levene's test. Gene abundance data and all soil data except inorganic N were then log-transformed to meet these assumptions. Pearson correlations were performed to analyse the relationships between gene abundances and soil chemical characteristics. A principal component analysis (PCA) was implemented to visualize how MFG abundances were distributed based on plantation type and sampling time. All statistical tests were considered significant at $P < 0.05$. SPSS version 22 was used for all statistical analyses except for PCA which was carried out in R (FactoMine and FactoExtra packages).

RESULTS

Soil chemical characteristics were significantly influenced by plantation type and sampling time, but the interaction of both factors was not significant, except for soil $\delta^{15}N$ (**Table 1**). Soil pH was higher under teak plantations than under flueggea or mixed

TABLE 1 | *P*-values obtained from a two-way analysis of variance (ANOVA) to detect the effects of plantation type, sampling time, and their interaction on the measured soil variables at Kolombangara, Solomon Islands.

	df	pH	Total C	Total N	C:N	NH_4^+-N	$\delta^{15}N$
Plantation type	2	$P = 0.000$	$P = 0.000$	$P = 0.000$	$P = 0.000$	$P = 0.053$	$P = 0.000$
Sampling time	2	$P = 0.030$	$P = 0.000$	$P = 0.001$	$P = 0.222$	$P = 0.068$	$P = 0.351$
Plantation type × sampling time	4	$P = 0.370$	$P = 0.141$	$P = 0.112$	$P = 0.681$	$P = 0.978$	$P = 0.006$

TABLE 2 | Soil chemical characteristics measured in different plantation types and at different sampling times at Kolombangara, Solomon Islands.

		pH (1:5 H_2O)	Total C (g kg^{-1})	Total N (g kg^{-1})
December 2012	Teak	6.15 (0.06) a B	60.11 (3.25) b	5.78 (0.33) b
	Flueggea	5.71 (0.14) b AB	75.38 (2.83) a A	6.84 (0.19) a A
	Mixed	5.72 (0.11) b	73.05 (3.33) a AB	6.46 (0.18) ab AB
May 2013	Teak	6.24 (0.04) a AB	52.02 (1.60) b	5.10 (0.18) b
	Flueggea	5.47 (0.13) b B	59.46 (3.45) ab B	5.36 (0.27) ab B
	Mixed	5.75 (0.13) b	66.60 (3.27) a B	6.01 (0.28) a B
December 2013	Teak	6.37 (0.06) a A	62.54 (3.40) b	6.03 (0.30) b
	Flueggea	5.96 (0.14) b A	69.96 (3.40) b A	6.31 (0.30) ab A
	Mixed	5.80 (0.10) b	86.00 (4.81) a A	7.47 (0.40) a A
		C:N	**NH_4^+-N (mg kg^{-1})**	**$\delta^{15}N$ (‰)**
December 2012	Teak	10.35 (0.14) b	238.22 (25.54)	6.68 (0.15) a
	Flueggea	11.00 (0.23) ab	258.21 (15.32)	4.04 (0.56) b
	Mixed	11.26 (0.29) a	247.87 (11.75)	5.46 (0.16) a
May 2013	Teak	10.23 (0.10) b	203.56 (15.19)	6.96 (0.09) a
	Flueggea	11.08 (0.27) a	219.08 (18.47)	4.60 (0.55) b
	Mixed	11.09 (0.20) a	228.95 (16.19)	5.28 (0.28) ab
December 2013	Teak	10.37 (0.14) b	202.56 (16.82)	6.47 (0.11) a
	Flueggea	11.12 (0.22) a	224.72 (12.05)	4.45 (0.51) b
	Mixed	11.51 (0.14) a	219.26 (13.92)	4.86 (0.18) ab

Values between brackets represent standard errors. Small letters indicate significant differences between plantation types for a same sampling time. Capital letters indicate significant differences between sampling times for a same plantation type. No small or capital letters indicate no significant differences between plantation types or sampling times respectively. All differences were considered significant at $P < 0.05$.

plantations, independent of sampling time (**Table 2**). Conversely, soil total C and N were lower under teak than under mixed plantations, except for total N in samples collected in December 2012. The lowest C:N ratios were also found under teak mono-plantations. The lowest $\delta^{15}N$ values were found in soil under flueggea mono-plantations, regardless of the time of sampling. The highest $\delta^{15}N$ values were found for teak plantation soil, although they did not significantly differ from mixed plantations. The effect of sampling time on soil pH, total C and total N was significant, and the highest values were generally found in the last sampling event (December 2013). No differences were found between treatments for soil NH_4^+ (**Table 2**).

The abundance of MFG involved in N cycling was significantly influenced by plantation type and sampling time, and the interaction of both factors was generally significant (**Table 3**). The abundance of total bacteria ranged from 1.27×10^8 to 1.27×10^9 copies ng^{-1} DNA and was significantly affected by plantation type in the first and last samplings (**Figure 2A**). The denitrification genes *narG* and *nosZ* were generally most abundant in soils of mixed plantations (**Figures 2B,E**). The abundance of *nirS* was the highest under teak mono-plantations in the first sampling, and under teak and flueggea mono-plantations in the second sampling. However, in the last sampling, *nirS* abundance was higher under mixed plantations than under mono-plantations (**Figure 2D**). The abundance of AOB was higher under teak plantations than under flueggea and mixed plantations in the first and last samplings (**Figure 2H**). No differences were found between plantation types in *nirK*, *nifH* and AOA, except in the second sampling when AOA abundance was the lowest under mixed plantations (**Figures 2C,F,G**).

Sampling time effects on the abundance of MFG were also detected (**Table 3**). The PCA biplot showed that sampling time was the main explanatory variable for gene abundance data, rather than plantation type (**Figures 3A,B**). Generally, the

TABLE 3 | *P*-values obtained from a two-way analysis of variance (ANOVA) to detect the effects of plantation type, sampling time, and their interaction on the abundances of the functional genes involved in soil N cycling at Kolombangara, Solomon Islands.

	df	*16S*	*narG*	*nirK*	*nirS*	*nosZ*	*nifH*	AOA	AOB
Plantation type	2	$P = 0.001$	$P = 0.007$	$P = 0.338$	$P = 0.000$	$P = 0.000$	$P = 0.650$	$P = 0.000$	$P = 0.031$
Sampling time	2	$P = 0.000$	$P = 0.934$	$P = 0.000$	$P = 0.000$	$P = 0.000$	$P = 0.262$	$P = 0.552$	$P = 0.000$
Plantation type × sampling time	4	$P = 0.000$	$P = 0.232$	$P = 0.059$	$P = 0.000$	$P = 0.004$	$P = 0.021$	$P = 0.254$	$P = 0.014$

abundances of *nirS* and *nirK* genes were higher in the last sampling event (December 2013) than in the first two samplings (**Figures 2C,D**). On the contrary, the abundances of genes 16S, *nosZ* and AOA were higher in the first sampling (December 2012) than in the two following sampling events (**Figures 2A,E,G**), although ANOVA results showed that this difference was not significant for AOA (Supplementary Table S2). The highest abundances for AOB were found in May 2013 (**Figure 2H**).

The relative abundances of *narG*, *nirK*, *nirS,* and *nosZ* within the total 16S rRNA gene showed that denitrification genes were generally more abundant under mixed plantations, independently from the sampling time (**Figures 4A–D**). The *nosZ*/(*nirK*+*nirS*) ratio was also larger under mixed plantations than under mono-plantations (**Figure 4E**). The relative abundances of the *nirK* and *nirS* genes revealed a sharp increase in the last sampling while the relative abundance of the *nosZ* gene significantly decreased in December 2013. As a result, the *nosZ*/(*nirK*+*nirS*) ratio was the lowest in the last sampling (**Figure 4E**). When expressed in relation to the size of the entire bacterial community, the *nifH* gene abundance varied depending on plantation type, being larger under mixed plantations than under mono-plantations in the first and last samplings (**Figure 4F**). The AOA/AOB ratio was also influenced by the sampling date, being the largest in the first sampling. In the first two samplings, the AOA/AOB ratio was larger under flueggea than under teak and mixed plantations. However, no significant differences were detected in the AOA/AOB ratio between plantation types in December 2013 (**Figure 4G**).

Pearson correlations showed that soil pH was positively related to the abundance of denitrification genes *nirK* and *nirS*, and to that of AOA (**Table 4**). Soil total C positively correlated with the abundance of total bacteria (16S rRNA), *nirK* and *nirS*, but negatively correlated with the abundance of AOA. Total N positively correlated with the abundance of most N-cycling genes except for *nosZ* and the nitrification genes (AOA and AOB). The C:N ratio strongly and positively correlated with the abundance of *nirK* and *nirS* and negatively correlated with the abundance of 16S rRNA and *nosZ*. Soil $\delta^{15}N$ only correlated with the abundance of AOA while NH_4^+ correlated positively with that of *narG* and nitrification genes AOA and AOB.

DISCUSSION

The objective of this study was to determine how the establishment of mixed plantations would influence soil N pools and transformations, measured through soil chemical properties and MFG abundance. Our results show that soil properties and MFG abundances were both influenced by plantation type. Inter-planting teak with flueggea decreased soil pH and may therefore reduce soil bacterial activities and nutrient cycling rates (Rousk et al., 2009), which could in turn lessen N losses from the system (Xu et al., 2013). Accelerations of N transformation rates and increases in N losses through leaching or denitrification have been associated with enriched soil ^{15}N signals (Bai et al., 2015; Reverchon et al., 2015), due to the discrimination against the heavier N isotope during microbially mediated N transformations (Högberg, 1997). Soil $\delta^{15}N$ values were significantly higher under teak than under flueggea plantation soils and intercropping teak with flueggea seemed to decrease soil $\delta^{15}N$, although not significantly, which indicates that N losses through volatilization, leaching, or denitrification could be reduced in mixed plantations.

Soil total C and N content increased when teak was inter-planted with flueggea, which translated into higher C:N ratios in soils from mixed plantations than those in teak mono-plantations. Soil C:N ratio is considered a good indicator of soil fertility as it reflects the coupling between soil organic C (SOC) and total N (Lou et al., 2012; Corral-Fernández et al., 2013). Higher C:N ratios may indicate a slowdown of SOC decomposition and N mineralisation as well as an enhancement of N immobilization (Davidson et al., 2003; Puget and Lal, 2005). Mixed plantations could therefore constitute a way to increase soil C and N stocks without losing the source of income that teak trees represent for local communities. Recently, Lang et al. (2014) showed that N acquisition and retention by subtropical trees were enhanced in mixed plantations when compared with those in mono-plantations, and that this increase could be observed as early as the sapling stage. Whether these positive effects will remain once the trees have reached their full height remains to be investigated, although evidences suggest that complementarity effects among co-occurring species, both at the above- and below-ground level, may promote resource-use efficiency in mixed species stands (Cardinale et al., 2007; Richards et al., 2010). Moreover, aging plantations have been shown to have a positive effect on SOC stocks due to an increase in tree productivity and C belowground allocation with time (Eclesia et al., 2012).

The abundance of MFG associated with N cycling was influenced by plantation type, although the sampling time effect seemed to be stronger than that of tree cover. This is consistent with findings by Yamamura et al. (2013) who reported that tree species scarcely influence N cycling genes and bacterial community structure in tropical plantations. The seasonal differences observed in the present study were unlikely due to variations in rainfall or temperature, since climatic conditions in Solomon Islands are relatively homogeneous through the year.

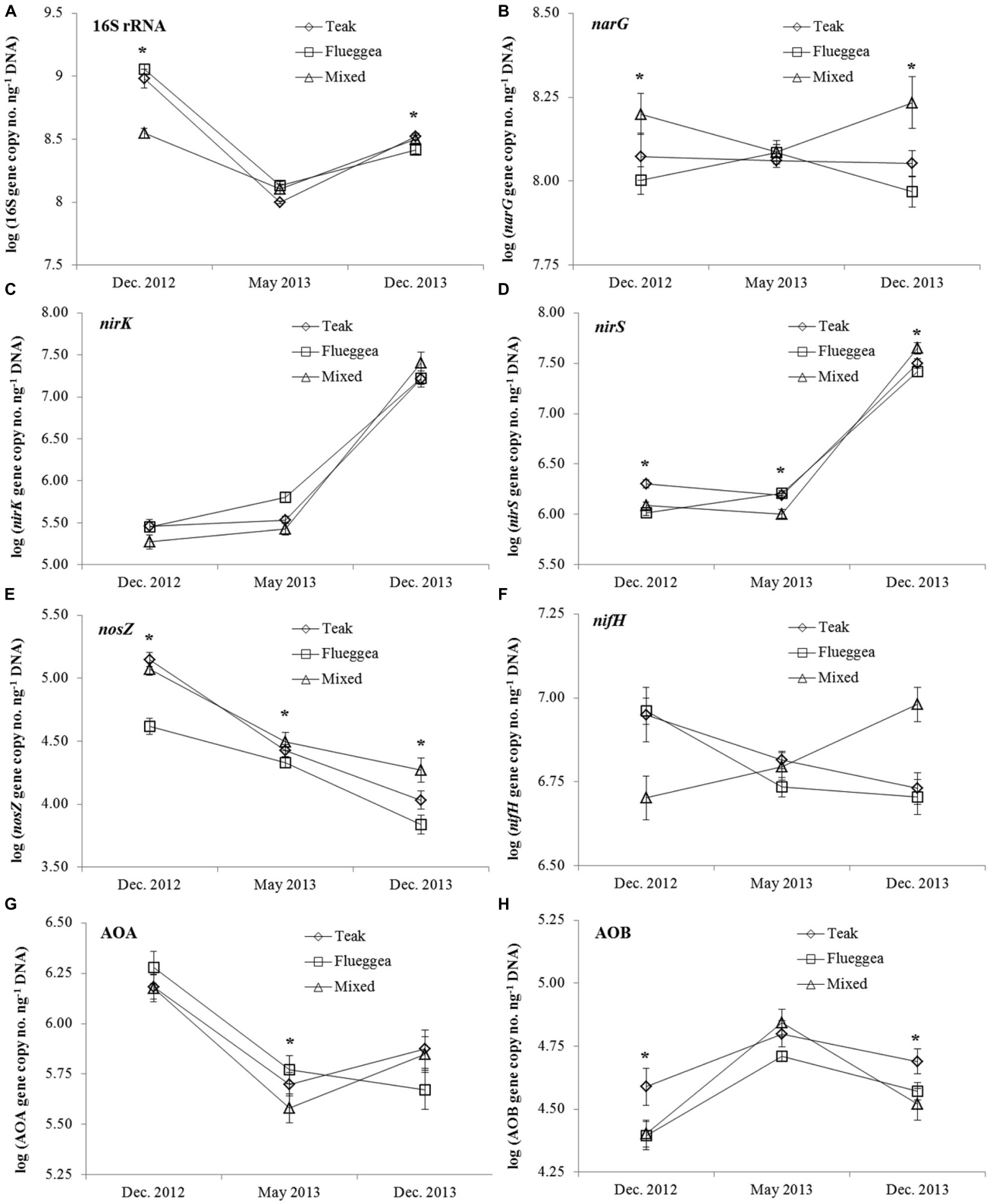

FIGURE 2 | Abundance of the total bacterial community (A) and of the *narG* (B), *nirK* (C), *nirS* (D), *nosZ* (E), *nifH* (F), AOA (G), and AOB (H) genes in soil under plantations of teak (diamonds), flueggea (squares), and mixed species (triangles) at three sampling dates. Gene abundances are expressed in log (gene copy numbers ng^{-1} DNA). Asterisks indicate significant differences between plantation types (Tukey HSD test, $P < 0.05$).

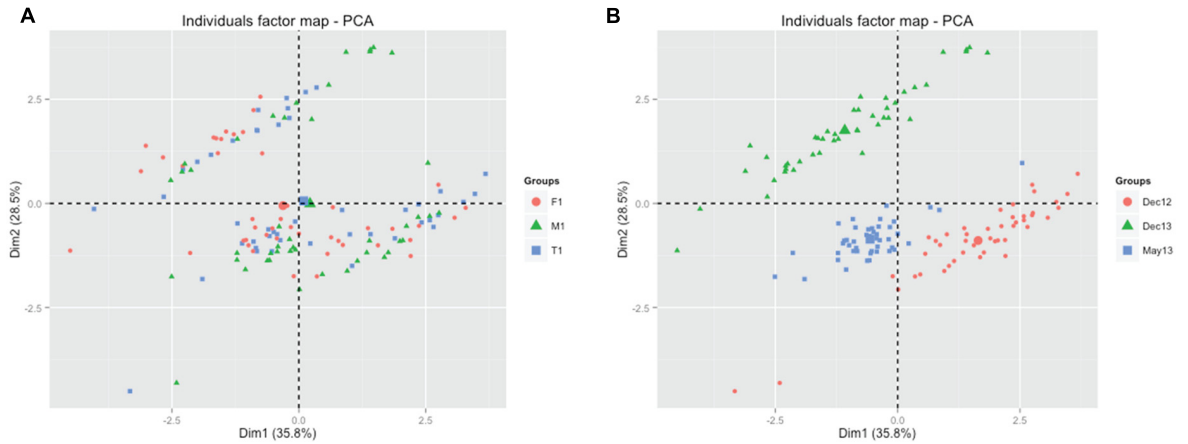

FIGURE 3 | Principal component analysis (PCA) biplot showing the clustering of microbial functional gene abundances per plantation type (A) and per sampling date (B). T: teak; F: flueggea; M: mixed; Dec12: December 2012; May13: May 2013; Dec13: December 2013.

However, seasonal variations in soil conditions, affecting plant growth and productivity, bacterial community composition, and ultimately ecosystem processes, could have altered microbial traits. Seasonal differences were detected in pH, total C and total N. Additionally, Rasche et al. (2011) showed that seasonal dynamics of microbial functional groups involved in N cycling were strongly related with seasonal changes in soil labile C and N, and in general with resource availability. This seasonality was especially apparent in the relative abundances of denitrification genes, with the relative abundance of nitrite reductase genes (*nirK* and *nirS*) showing a drastic increase in the last sampling. As a consequence, the *nosZ*/(*nirK*+*nirS*) ratio presented a sharp decrease at the same period, as did the AOA/AOB ratio. Denitrifier and nitrifier microorganisms (bacteria and archaea) have been shown to be particularly affected by seasonal shifts in the soil environment, such as soil temperature or moisture (Shen et al., 2008; Stres et al., 2008), which are closely related with resource availability and labile C pools (Bell et al., 2009). Seasonal patterns of tree belowground C allocation may also contribute to temporal variations in microbial dynamics (Kaiser et al., 2011; Churchland and Grayston, 2014). More recently, Graham et al. (2014) reported that temporal dynamics were critical to unraveling the relationships between soil properties and gene abundance data, and thus recommended to examine seasonal samples separately in order to develop accurate models of ecosystem functioning.

Generally, the abundance of MFG associated with denitrification (*narG*, *nosZ*, and *nirS*) was larger in soils from mixed plantations than in those from teak mono-plantations, especially in the last sampling. Gene copy numbers in MFG only vary from 1 to 3 in bacterial cells, while the number of 16S rRNA gene copies range from 1 to 13 per cell (Fogel et al., 1999). Therefore, whilst the observed differences in 16S rRNA gene abundance between tree plantation systems could have been due to differences in species composition of the bacterial community (Liu et al., 2015), our results indicate that denitrifier communities were more abundant under mixed plantations, which is confirmed by the relative abundances of denitrification genes. An increase in the abundance of *narG* (nitrate reductase) and *nosZ* (nitrous oxide reductase) may reduce the negative environmental impacts associated with leaching of NO_3^- and with N_2O emissions (Henry et al., 2006). The larger *nosZ*/(*nirK*+*nirS*) ratio under mixed plantations indicated an enrichment in *nosZ*-harboring denitrifiers when teak was inter-planted with flueggea, which could in turn have implications for N_2O/N_2 emissions from tree plantations (Liu et al., 2015). The measurement of atypical *nosZ* abundance, which was not quantified in this study, would also complement the analysis of bacterial and archeal contributions to N_2O emissions from plantation soils (Sanford et al., 2012).

Overall, the increase in denitrification gene abundances in mixed plantations may indicate larger denitrifying bacterial communities when teak is inter-planted with flueggea (Baudoin et al., 2009). Denitrifier bacteria have been reported to be influenced by the quantity and composition of organic compounds emitted by roots or resulting from the decomposition of organic residues (Kandeler et al., 2006; Henry et al., 2008). Mixed plantations, by promoting the diversity of bioavailable C sources associated with root exudates and litterfall decomposition, could sustain more abundant denitrifier communities than mono-plantations. The abundance of most denitrification genes correlated with soil total N content and C:N ratio, and *nirK*/*nirS* gene abundances positively correlated with total C content. These findings are consistent with results from previous studies (Lindsay et al., 2010; Ducey et al., 2013) and confirm the heterotrophic nature of denitrifier bacterial communities (Baudoin et al., 2009). Furthermore, soil total C, total N, and C:N were all influenced by plantation type, which suggests that tree species mixture is likely to be a significant driver of the denitrifier community through the modification of soil properties.

No differences in *nifH* abundance were detected between plantation types. This is consistent with findings from Rachid

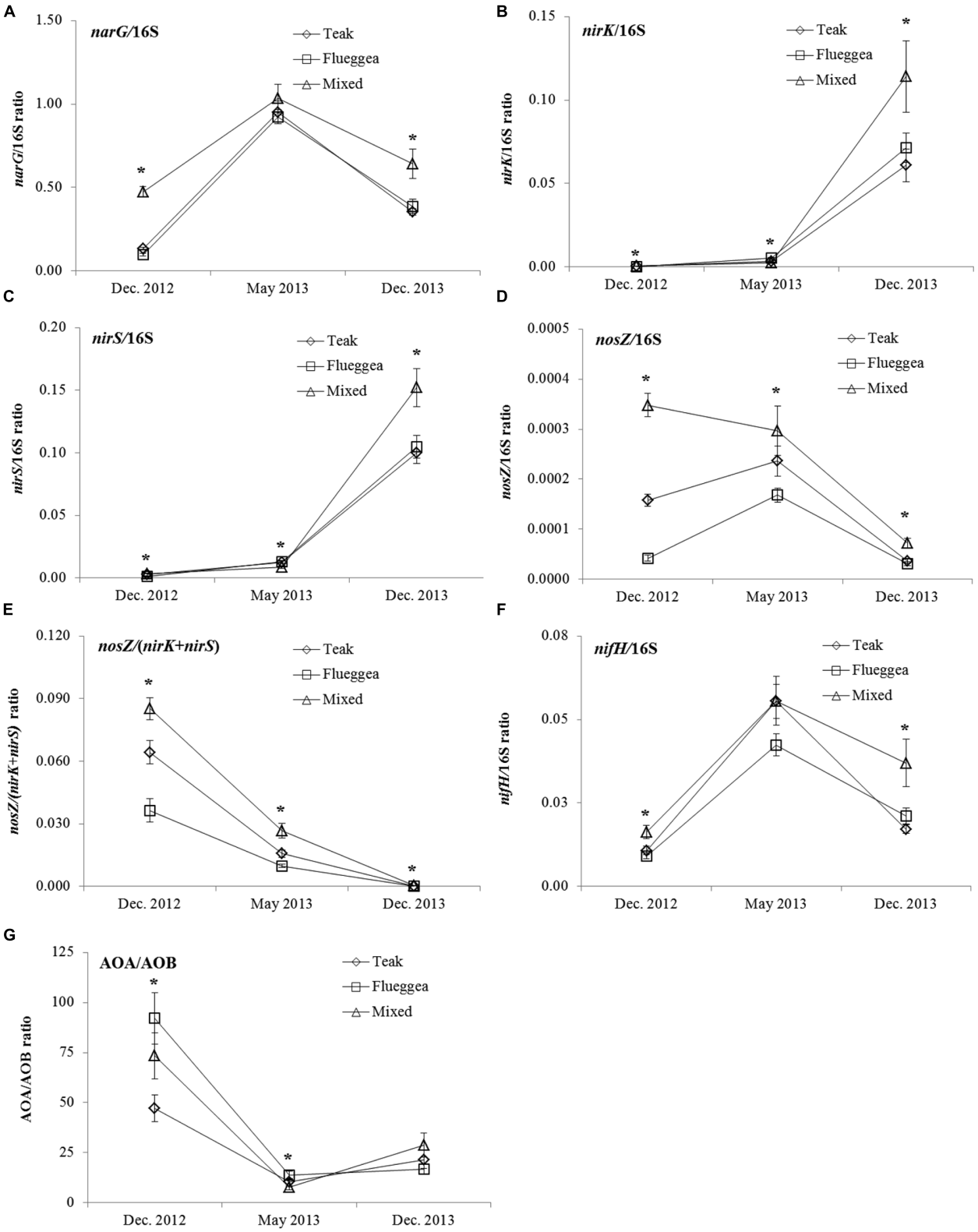

FIGURE 4 | Relative abundance of the *narG* (A), *nirK* (B), *nirS* (C), *nosZ* (D) genes, nosZ/(nirK+nirS) ratio (E), relative abundance of the *nifH* gene (F), and AOA/AOB ratio (G) in soil under plantations of teak (diamonds), flueggea (squares), and mixed species (triangles) at three sampling dates. Asterisks indicate significant differences between plantation types (Tukey HSD test, $P < 0.05$).

TABLE 4 | Pearson coefficients for correlation between functional gene abundances and soil characteristics, Kolombangara, Solomon Islands.

	16S	*narG*	*nirK*	*nirS*	*nosZ*	*nifH*	*AOA*	*AOB*
pH	0.045	0.042	0.204*	0.217*	−0.066	−0.077	0.410**	0.044
C	0.223**	0.159	0.209*	0.207*	0.001	0.168	−0.206*	−0.133
N	0.277**	0.181*	0.219*	0.209*	0.025	0.190*	−0.149	−0.12
C:N	−0.332*	−0.096	0.307**	0.308**	−0.329**	−0.087	−0.139	0.105
$\delta^{15}N$	−0.066	0.107	−0.027	0.002	0.129	−0.037	0.228**	0.102
NH_4^+	−0.028	0.170*	0.116	0.088	0.016	0.143	0.299**	0.259**

*Represents significance at $P < 0.05$; **represents significance at $P < 0.01$.

et al. (2013) who showed that soil free-living nitrogen-fixing bacteria were not altered by the establishment of mixed plantations. However, in the first and last samplings, the relative abundance of the *nifH* gene was larger in mixed plantations than in mono-plantations, which reflects an enrichment in N-fixing bacteria when teak was inter-planted with flueggea and could, over time, increase soil N content (Lammel et al., 2015). Nitrifier communities were also affected by plantation type, as shown by the significant decrease in AOB abundance under mixed plantations compared with teak mono-plantations. This reduction of AOB abundance in mixed plantations did not translate into alterations in soil NH_4^+ concentrations, although both AOA and AOB genes correlated significantly with soil NH_4^+. The lack of alteration in soil NH_4^+ may be due to the dominance of AOA in the ammonia oxidation process, as indicated by AOA:AOB ratios higher than 10 in almost all treatments (Prosser and Nicol, 2012). Furthermore, the abundance and community structure of nitrifiers have been shown to be highly dependent upon pH (Wessén et al., 2011; Levy-Booth et al., 2014), and different phylotypes of bacterial and archaeal ammonia oxidisers are selected in soils with different pH (Nicol et al., 2008), which in turn could affect nitrification rates.

Differences in N pools and transformations between different tree plantation systems may be attributed to a combination of alterations in the soil physico-chemical environment and in soil microbial communities (Anderson et al., 2014). Tree mycorrhizal symbionts could further influence N cycling by accessing different soil N sources or by enhancing the immobilization of inorganic N (Michelsen et al., 1996). While teak has been reported to form arbuscular mycorrhizal fungal associations (Rajan et al., 2000), the mycorrhizal status of flueggea is yet to be studied and may have implications for species interactions in these mixed plantations. Moreover, bacterial taxonomic and functional diversity could be affected by tree species composition directly through changes in the amount and composition of root exudates and litterfall, and indirectly through changes in soil parameters (da C Jesus et al., 2009; Rousk et al., 2010). The significant correlations found in the present study between some soil factors and MFG abundances confirmed the influence of plantation type on soil bacterial communities through the modification of the soil environment. However, there was no strong coupling between gene abundances and the measured soil parameters. This lack of correspondence may be due to different factors. First, process rates may be more readily influenced by changes in tree cover than taxonomic or functional diversity of bacterial communities (Rocca et al., 2015). Moreover, changes in soil parameters may have a stronger effect on the relative proportion of nitrifier/denitrifier bacteria than on MFG abundance (Anderson et al., 2014). Finally, the presence of functional genes does not always indicate an active bacterial community (Levy-Booth et al., 2014) and expression of MFG measured through mRNA transcripts, rather than MFG abundance, could be a better predictor of microbial functional capacity and hence N transformation rates in soil (Wood et al., 2015).

Due to the lack of proper conditions available at the sampling site, it was not possible to freeze the samples and maintain them at −80°C until DNA extraction. Sample storage at 4°C for up to 5 days prior to analysis may have induced changes in bacterial community structure and MFG abundances, as reported by Ott et al. (2004) for fecal samples. However, recent findings by Lauber et al. (2010) and Rubin et al. (2013) showed that neither storage time nor storage temperature drastically affected microbial community composition and structure from soil samples. In addition, storage conditions were consistent across our three sampling events, thereby reducing any bias resulting from sample collection and preparation.

With teak native forests disappearing, teak plantations are expanding and now cover $4.3{\cdot}10^6$ ha (Fernández-Moya et al., 2014). Establishing mixed plantations as alternatives to mono-species teak stands would guarantee economic returns without a subsequent impoverishment of soil fertility. Our results show that inter-planting teak with flueggea would benefit soil quality and sustainability by increasing soil C and N pools, which we hypothesized could be due to a decrease in potential losses from leaching and N_2O emissions or to higher inputs from flueggea trees. In addition, soil quality under mixed plantations would be enhanced by the diversity of bioavailable C compounds from root exudates and leaf litter. Kirby and Potvin (2007) reported a positive correlation between biodiversity and organic C in soils, thereby suggesting that mixed plantations could improve soil physical structure (Xing et al., 2004). Our findings are further supported by productivity and aboveground nutrient accumulation data showing the potential for teak to be grown in mixed plantations (Vigulu, 2015). Further research should concentrate on assessing other mixed plantation models under different sets of environmental conditions, and on incorporating microbial dynamics into plantation management to improve productivity while mitigating soil fertility loss.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01439

Figure S1 | Average rainfall data at Kolombangara, Western Province, Solomon Islands, from 1993 to 2014.

REFERENCES

Anderson, C. R., Hamonts, K., Clough, T. J., and Condron, L. M. (2014). Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. *Agric. Ecosyst. Environ.* 191, 63–72. doi: 10.1016/j.agee.2014.02.021

Bai, S. H., Blumfield, T. J., Xu, Z., Chen, C., and Wild, C. H. (2012). Soil organic matter dynamics and nitrogen availability in response to site preparation and management during revegetation in tropical Central Queensland, Australia. *J. Soil Sediments* 12, 386–395. doi: 10.1007/s11368-011-0466-9

Bai, S. H., Xu, C. Y., Xu, Z., Blumfield, T. J., Zhao, H., Wallace, H., et al. (2015). Soil and foliar nutrient and nitrogen isotope composition (δ15N) at 5 years after poultry litter and green waste biochar amendment in a macadamia orchard. *Environ. Sci. Pollut. Res. Int.* 22, 3803–3809. doi: 10.1007/s11356-014-3649-2

Balieiro, F. D. C., Pereira, M. G., Alves, B. J. R., Resende, A. S. D., and Franco, A. A. (2008). Soil carbon and nitrogen in pasture soil reforested with *Eucalyptus* and Guachapele. *Rev. Bras. Ciênc. Solo* 32, 1253–1260. doi: 10.1590/S0100-06832008000300033

Baudoin, E., Philippot, L., Cheneby, D., Chapuis-Lardy, L., Fromin, N., Bru, D., et al. (2009). Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil. *Soil Biol. Biochem.* 41, 1703–1709. doi: 10.1016/j.soilbio.2009.05.015

Bell, C. W., Acosta-Martinez, V., McIntyre, N. E., Cox, S., Tissue, D. T., and Zak, J. C. (2009). Linking microbial community structure and function to seasonal differences in soil moisture and temperature in a Chihuahuan desert grassland. *Microb. Ecol.* 58, 827–842. doi: 10.1007/s00248-009-9529-5

Bissett, A., Brown, M. V., Siciliano, S. D., and Thrall, P. H. (2013). Microbial community responses to anthropogenically induced environmental change: towards a systems approach. *Ecol. Lett.* 16, 128–139. doi: 10.1111/ele.12109

Bissett, A., Richardson, A. E., Baker, G., and Thrall, P. H. (2011). Long-term land use effects on soil microbial community structure and function. *Appl. Soil Ecol.* 51, 66–78. doi: 10.1016/j.apsoil.2011.08.010

Cardinale, B. J., Wright, J. P., Cadotte, M. W., Carroll, I. T., Hector, A., Srivastava, D. S., et al. (2007). Impacts of plant diversity on biomass production increase through time because of species complementarity. *Proc. Natl. Acad. Sci. U. S. A.* 104, 18123–18128. doi: 10.1073/pnas.0709069104

Chen, C. R., Xu, Z. H., Blumfield, T. J., and Hughes, J. M. (2003). Soil microbial biomass during the early establishment of hoop pine plantation: seasonal variation and impacts of site preparation. *For. Ecol. Manage.* 186, 213–225. doi: 10.1016/S0378-1127(03)00275-5

Churchland, C., and Grayston, S. J. (2014). Specificity of plant-microbe interactions in the tree mycorrhizosphere biome and consequences for soil C cycling. *Front. Microbiol.* 5:261. doi: 10.3389/fmicb.2014.00261

Corral-Fernández, R., Parras-Alcántara, L., and Lozano-García, B. (2013). Stratification ratio of soil organic C, N and C: N in Mediterranean evergreen oak woodland with conventional and organic tillage. *Agric. Ecosyst. Environ.* 164, 252–259. doi: 10.1016/j.agee.2012.11.002

Correa-Galeote, D., Marco, D. E., Tortosa, G., Bru, D., Philippot, L., and Bedmar, E. J. (2013). Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments. *FEMS Microbiol. Ecol.* 83, 340–351. doi: 10.1111/j.1574-6941.2012.01479.x

Čuhel, J., Šimek, M., Laughlin, R. J., Bru, D., Chèneby, D., Watson, C. J., et al. (2010). Insights into the effect of soil pH on N2O and N2 emissions and denitrifier community size and activity. *Appl. Environ. Microbiol.* 76, 1870–1878. doi: 10.1128/AEM.02484-09

da C Jesus, E., Marsh, T. L., Tiedje, J. M., and de S Moreira, F. M. (2009). Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME J.* 3, 1004–1011. doi: 10.1038/ismej.2009.47

Davidson, E. A., Chorover, J., and Dail, D. B. (2003). A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis. *Glob. Change Biol.* 9, 228–236. doi: 10.1046/j.1365-2486.2003.00592.x

Ducey, T. F., Ippolito, J. A., Cantrell, K. B., Novak, J. M., and Lentz, R. D. (2013). Addition of activated switchgrass biochar to an aridic subsoil increases microbial nitrogen cycling gene abundances. *Appl. Soil Ecol.* 65, 65–72. doi: 10.1016/j.apsoil.2013.01.006

Eclesia, R. P., Jobbagy, E. G., Jackson, R. B., Biganzoli, F., and Piñeiro, G. (2012). Shifts in soil organic carbon for plantation and pasture establishment in native forests and grasslands of South America. *Glob. Change Biol.* 18, 3237–3251. doi: 10.1111/j.1365-2486.2012.02761.x

Fernández-Moya, J., Alvarado, A., Forsythe, W., Ramírez, L., Algeet-Abarquero, N., and Marchamalo-Sacristán, M. (2014). Soil erosion under teak (*Tectona grandis* Lf) plantations: general patterns, assumptions and controversies. *Catena* 123, 236–242. doi: 10.1016/j.catena.2014.08.010

Fogel, G. B., Collins, C. R., Li, J., and Brunk, C. F. (1999). Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population. *Microb. Ecol.* 38, 93–113. doi: 10.1007/s002489900162

Graham, E. B., Wieder, W. R., Leff, J. W., Weintraub, S. R., Townsend, A. R., Cleveland, C. C., et al. (2014). Do we need to understand microbial communities to predict ecosystem function? A comparison of statistical models of nitrogen cycling processes. *Soil Biol. Biochem.* 68, 279–282. doi: 10.1016/j.soilbio.2013.08.023

Hallin, S., Jones, C. M., Schloter, M., and Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J.* 3, 597–605. doi: 10.1038/ismej.2008.128

He, Y., Xu, Z., Chen, C., Burton, J., Ma, Q., Ge, Y., et al. (2008). Using light fraction and macroaggregate associated organic matters as early indicators for management-induced changes in soil chemical and biological properties in adjacent native and plantation forests of subtropical Australia. *Geoderma* 147, 116–125. doi: 10.1016/j.geoderma.2008.08.002

Henry, S., Baudouin, E., López-Gutiérrez, J. C., Martin-Laurent, F., Brauman, A., and Philippot, L. (2004). Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *J. Microbiol. Methods* 59, 327–335. doi: 10.1016/j.mimet.2004.07.002

Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Appl. Environ. Microbiol.* 72, 5181–5189. doi: 10.1128/AEM.00231-06

Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Chèneby, D., et al. (2008). Disentangling the rhizosphere effect on nitrate reducers and denitrifiers:

insight into the role of root exudates. *Environ. Microbiol.* 10, 3082–3092. doi: 10.1111/j.1462-2920.2008.01599.x

Hietz, P., Turner, B. L., Wanek, W., Richter, A., Nock, C. A., and Wright, S. J. (2011). Long-term change in the nitrogen cycle of tropical forests. *Science* 334, 664–666. doi: 10.1126/science.1211979

Högberg, P. (1997). Tansley review no. 95: 15N natural abundance in soil-plant systems. *New Phytol.* 137, 179–203. doi: 10.1046/j.1469-8137.1997.00808.x

Kadambi, K. (1972). Silviculture and management of teak. *Bulletin* 24, 137.

Kaiser, C., Fuchslueger, L., Koranda, M., Gorfer, M., Stange, C. F., Kitzler, B., et al. (2011). Plants control the seasonal dynamics of microbial N cycling in a beech forest soil by belowground C allocation. *Ecology* 92, 1036–1051. doi: 10.1890/10-1011.1

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., and Philippot, L. (2006). Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* 72, 5957–5962. doi: 10.1128/AEM.00439-06

Kirby, K. R., and Potvin, C. (2007). Variation in carbon storage among tree species: implications for the management of a small-scale carbon sink project. *For. Ecol. Manage.* 246, 208–221. doi: 10.1016/j.foreco.2007.03.072

Kourtev, P. S., Ehrenfeld, J. G., and Haggblom, M. (2003). Experimental analysis of the effect of exotic and native plant species on the structure and function of soil microbial communities. *Soil Biol. Biochem.* 35, 895–905. doi: 10.1016/S0038-0717(03)00120-2

Lacombe, S., Bradley, R. L., Hamel, C., and Beaulieu, C. (2009). Do tree-based intercropping systems increase the diversity and stability of soil microbial communities? *Agric. Ecosyst. Environ.* 131, 25–31. doi: 10.1016/j.agee.2008.08.010

Ladrach, W. (2009). *Management of Teak Plantations for Solid Wood Products. International Society of Tropical Foresters News, Special Report*. Available at: http://www.istf-bethesda.org/specialreports/teca_teak/teak.pdf (accessed December 2009).

Lammel, D. R., Feigl, B. J., Cerri, C. C., and Nüsslein, K. (2015). Specific microbial gene abundances and soil parameters contribute to C, N, and greenhouse gas process rates after land use change in Southern Amazonian Soils. *Front. Microbiol.* 6:1057. doi: 10.3389/fmicb.2015.01057

Lang, A. C., Oheimb, G., Scherer-Lorenzen, M., Yang, B., Trogisch, S., Bruelheide, H., et al. (2014). Mixed afforestation of young subtropical trees promotes nitrogen acquisition and retention. *J. Appl. Ecol.* 51, 224–233. doi: 10.1111/1365-2664.12157

Lauber, C. L., Zhou, N., Gordon, J. I., Knight, R., and Fierer, N. (2010). Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiol. Lett.* 307, 80–86. doi: 10.1111/j.1574-6968.2010.01965.x

Levy-Booth, D. J., Prescott, C. E., and Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biol. Biochem.* 75, 11–25. doi: 10.1016/j.soilbio.2014.03.021

Levy-Booth, D. J., and Winder, R. S. (2010). Quantification of nitrogen reductase and nitrite reductase genes in soil of thinned and clear-cut Douglas-fir stands by using real-time PCR. *Appl. Environ. Microbiol.* 76, 7116–7125. doi: 10.1128/AEM.02188-09

Lindsay, E. A., Colloff, M. J., Gibb, N. L., and Wakelin, S. A. (2010). The abundance of microbial functional genes in grassy woodlands is influenced more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. *Appl. Environ. Microbiol.* 76, 5547–5555. doi: 10.1128/AEM.03054-09

Liu, X., Chen, C., Wang, W., Hughes, J. M., Lewis, T., Hou, E., et al. (2015). Vertical distribution of soil denitrifying communities in a wet sclerophyll forest under long-term repeated burning. *Microb. Ecol.* 70, 993–1003. doi: 10.1007/s00248-015-0639-y

Liu, X., Chen, C. R., Wang, W. J., Hughes, J. M., Lewis, T., Hou, E. Q., et al. (2013). Soil environmental factors rather than denitrification gene abundance control N2O fluxes in a wet sclerophyll forest with different burning frequency. *Soil Biol. Biochem.* 57, 292–300. doi: 10.1016/j.soilbio.2012.10.009

Lou, Y., Xu, M., Chen, X., He, X., and Zhao, K. (2012). Stratification of soil organic C, N and C: N ratio as affected by conservation tillage in two maize fields of China. *Catena* 95, 124–130. doi: 10.1016/j.catena.2012.02.009

Michelsen, A., Schmidt, I. K., Jonasson, S., Quarmby, C., and Sleep, D. (1996). Leaf 15N abundance of subarctic plants provides field evidence that ericoid, ectomycorrhizal and non-and arbuscular mycorrhizal species access different sources of soil nitrogen. *Oecologia* 105, 53–63. doi: 10.1007/BF00328791

Miranda, I., Sousa, V., and Pereira, H. (2011). Wood properties of teak (*Tectona grandis*) from a mature unmanaged stand in East Timor. *J. Wood Sci.* 57, 171–178. doi: 10.1007/s10086-010-1164-8

Montagnini, F. (2000). Accumulation in above-ground biomass and soil storage of mineral nutrients in pure and mixed plantations in a humid tropical lowland. *For. Ecol. Manage.* 134, 257–270. doi: 10.1016/S0378-1127(99)00262-5

Nicol, G. W., Leininger, S., Schleper, C., and Prosser, J. I. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ. Microbiol.* 10, 2966–2978. doi: 10.1111/j.1462-2920.2008.01701.x

Ott, S. J., Musfeldt, M., Timmis, K. N., Hampe, J., Wenderoth, D. F., and Schreiber, S. (2004). In vitro alterations of intestinal bacterial microbiota in fecal samples during storage. *Diagn. Microbiol. Infect. Dis.* 50, 237–245. doi: 10.1016/j.diagmicrobio.2004.08.012

Prosser, J. I., and Nicol, G. W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends Microbiol.* 20, 523–531. doi: 10.1016/j.tim.2012.08.001

Puget, P., and Lal, R. (2005). Soil organic carbon and nitrogen in a Mollisol in central Ohio as affected by tillage and land use. *Soil Tillage Res.* 80, 201–213. doi: 10.1016/j.still.2004.03.018

Rachid, C. T. C. C., Balieiro, F. C., Peixoto, R. S., Pinheiro, Y. A. S., Piccolo, M. C., Chaer, G. M., et al. (2013). Mixed plantations can promote microbial integration and soil nitrate increases with changes in the N cycling genes. *Soil Biol. Biochem.* 66, 146–153. doi: 10.1016/j.soilbio.2013.07.005

Rajan, S. K., Reddy, B. J. D., and Bagyaraj, D. J. (2000). Screening of arbuscular mycorrhizal fungi for their symbiotic efficiency with *Tectona grandis*. *For. Ecol. Manage.* 126, 91–95. doi: 10.1016/S0378-1127(99)00089-4

Rasche, F., Knapp, D., Kaiser, C., Koranda, M., Kitzler, B., Zechmeister-Boltenstern, S., et al. (2011). Seasonality and resource availability control bacterial and archaeal communities in soils of a temperate beech forest. *ISME J.* 5, 389–402. doi: 10.1038/ismej.2010.138

Reverchon, F., Flicker, R. C., Yang, H., Yan, G., Xu, Z., Chen, C., et al. (2014). Changes in δ15N in a soil–plant system under different biochar feedstocks and application rates. *Biol. Fertil. Soils* 50, 275–283. doi: 10.1007/s00374-013-0850-2

Reverchon, F., Yang, H., Ho, T. Y., Yan, G., Wang, J., Xu, Z., et al. (2015). A preliminary assessment of the potential of using an acacia—biochar system for spent mine site rehabilitation. *Environ. Sci. Pollut. Res. Int.* 22, 2138–2144. doi: 10.1007/s11356-014-3451-1

Richards, A. E., Forrester, D. I., Bauhus, J., and Scherer-Lorenzen, M. (2010). The influence of mixed tree plantations on the nutrition of individual species: a review. *Tree Physiol.* 30, 1192–1208. doi: 10.1093/treephys/tpq035

Rocca, J. D., Hall, E. K., Lennon, J. T., Evans, S. E., Waldrop, M. P., Cotner, J. B., et al. (2015). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. *ISME J.* 9, 1693–1699. doi: 10.1038/ismej.2014.252

Rotthauwe, J., Witzel, K., and Liesack, W. (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63, 4704–4712.

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., et al. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J.* 4, 1340–1351. doi: 10.1038/ismej.2010.58

Rousk, J., Brookes, P. C., and Bååth, E. (2009). Contrasting soil pH effects on fungal and bacterial growth suggests functional redundancy in carbon mineralisation. *Appl. Environ. Microbiol.* 75, 1589–1596. doi: 10.1128/AEM.02775-08

Rubin, B. E., Gibbons, S. M., Kennedy, S., Hampton-Marcell, J., Owens, S., and Gilbert, J. A. (2013). Investigating the impact of storage conditions on microbial community composition in soil samples. *PLoS ONE* 8:e70460. doi: 10.1371/journal.pone.0070460

Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-García, C., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19709–19714. doi: 10.1073/pnas.1211238109

Severin, I., and Stal, L. J. (2010). nifH expression by five groups of phototrophs compared with nitrogenase activity in coastal microbial mats. *FEMS Microbiol. Ecol.* 73, 55–67. doi: 10.1111/j.1574-6941.2010.00875.x

Shen, J. P., Zhang, L. M., Zhu, Y. G., Zhang, J. B., and He, J. Z. (2008). Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environ. Microbiol.* 10, 1601–1611. doi: 10.1111/j.1462-2920.2008.01578.x

Stres, B., Danevčič, T., Pal, L., Fuka, M. M., Resman, L., Leskovec, S., et al. (2008). Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. *FEMS Microbiol. Ecol.* 66, 110–122. doi: 10.1111/j.1574-6941.2008.00555.x

Thevathasan, N. V., and Gordon, A. M. (2004). Ecology of tree intercropping systems in the North temperate region: experiences from southern Ontario, Canada. *Agrofor. Syst.* 61, 257–268. doi: 10.1023/B:AGFO.0000029003.00933.6d

Thomson, L. A. J. (2006). "Flueggea flexuosa (poumuli) ver. 2.1," in *Species Profiles for Pacific Island Agroforestry*, ed. C. R. Elevitch (Holualoa, HI: Permanent Agriculture Resources).

Vigulu, V. W. (2015). *Intra-Specific Interactions in Tropical Agroforestry Systems*. Ph. D. dissertation, Griffith University, Nathan, QLD.

Wessén, E., Söderström, M., Stenberg, M., Bru, D., Hellman, M., Welsh, A., et al. (2011). Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. *ISME J.* 5, 1213–1225. doi: 10.1038/ismej.2010.206

Wieder, W. R., Cleveland, C. C., Taylor, P. G., Nemergut, D. R., Hinckley, E. L., Philippot, L., et al. (2013). Experimental removal and addition of leaf litter inputs reduces nitrate production and loss in a lowland tropical forest. *Biogeochemistry* 113, 629–642. doi: 10.1007/s10533-012-9793-1

Wolfe, B. T., Dent, D. H., Deago, J., and Wishnie, M. H. (2015). Forest regeneration under *Tectona grandis* and *Terminalia amazonia* plantation stands managed for biodiversity conservation in western Panama. *New Forest.* 46, 157–165. doi: 10.1007/s11056-014-9448-2

Wood, S. A., Almaraz, M., Bradford, M. A., McGuire, K. L., Naeem, S., Neill, C., et al. (2015). Farm management, not soil microbial diversity, controls nutrient loss from smallholder tropical agriculture. *Front. Microbiol.* 6:90. doi: 10.3389/fmicb.2015.00090

Xing, B., Liu, X., Liu, J., and Han, X. (2004). Physical and chemical characteristics of a typical mollisol in China. *Commun. Soil Sci. Plant Anal.* 35, 1829–1838. doi: 10.1081/LCSS-200026802

Xu, Y. B., Xu, Z. H., Cai, Z. C., and Reverchon, F. (2013). Review of denitrification in tropical and subtropical soils of terrestrial ecosystems. *J. Soil Sediments* 13, 699–710. doi: 10.1007/s11368-013-0650-1

Yamamura, T., Schwendenmann, L., and Lear, G. (2013). Tree species identity has little impact on the structure of soil bacterial communities in a 10-year-old tropical tree plantation. *Biol. Fertil. Soils* 49, 819–828. doi: 10.1007/s00374-013-0774-x

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Farm management, not soil microbial diversity, controls nutrient loss from smallholder tropical agriculture

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Tropical smallholder agriculture is undergoing rapid transformation in nutrient cycling pathways as international development efforts strongly promote greater use of mineral fertilizers to increase crop yields. These changes in nutrient availability may alter the composition of microbial communities with consequences for rates of biogeochemical processes that control nutrient losses to the environment. Ecological theory suggests that altered microbial diversity will strongly influence processes performed by relatively few microbial taxa, such as denitrification and hence nitrogen losses as nitrous oxide, a powerful greenhouse gas. Whether this theory helps predict nutrient losses from agriculture depends on the relative effects of microbial community change and increased nutrient availability on ecosystem processes. We find that mineral and organic nutrient addition to smallholder farms in Kenya alters the taxonomic and functional diversity of soil microbes. However, we find that the direct effects of farm management on both denitrification and carbon mineralization are greater than indirect effects through changes in the taxonomic and functional diversity of microbial communities. Changes in functional diversity are strongly coupled to changes in specific functional genes involved in denitrification, suggesting that it is the expression, rather than abundance, of key functional genes that can serve as an indicator of ecosystem process rates. Our results thus suggest that widely used broad summary statistics of microbial diversity based on DNA may be inappropriate for linking microbial communities to ecosystem processes in certain applied settings. Our results also raise doubts about the relative control of microbial composition compared to direct effects of management on nutrient losses in applied settings such as tropical agriculture.

Keywords: carbon mineralization, denitrification, fertilization, GeoChip, microbial diversity, smallholder agriculture, tropics

INTRODUCTION

Agricultural management, such as mineral nutrient addition, can lead to marked changes in the taxonomic composition of soil microbial communities (Ramirez et al., 2010, 2012; Fierer et al., 2011; Wood et al., 2015). The pairing of mineral and organic nutrient addition to agriculture can significantly impact the ability of soil microbial communities to catabolize a range of carbon (C) substrates as well as affect the abundance of microbial functional genes involved in multiple aspects of C, nitrogen (N), and phosphorus (P) cycling (Wood et al., 2015). Some of the microbially driven processes associated with these changes in functional capacity, such as denitrification and decomposition, determine the retention and loss of nutrients in ecosystems and are thus important to managing agriculture for crop production while minimizing nutrient losses to the environment (Vitousek et al., 2009). There is thus keen interest in whether changes in microbial community composition can directly impact rates of ecosystem processes (e.g., Wessén et al., 2011; Wallenstein and Hall, 2012; Philippot et al., 2013; van der Heijden and Wagg, 2013; Krause et al., 2014).

Certain ecosystem processes are likely to be more sensitive to changes in microbial community composition than others. Narrow processes are most likely to be affected by changes in community composition because they require a specific physiological pathway and/or are carried out by a phylogenetically clustered group of organisms (Schimel and Schaeffer, 2012). Thus, processes can be either physiologically narrow, phylogenetically narrow, or both. In this manuscript we use the term "narrow" to refer to physiologically narrow processes that require specific physiological pathways, regardless of their distribution in

the microbial phylogeny. For instance, we refer to denitrification as a narrow process because it requires particular genes that code for enzymes capable of reducing various forms of nitrogen. Because a relatively small proportion of microorganisms carry these genes, changes in community composition that lead to a shift in the relative abundance of denitrifiers—or changes in the abundances of the relevant functional genes—should have significant impacts on rates of denitrification (Pett-Ridge and Firestone, 2005; Philippot et al., 2013; Powell et al., 2015). Mineralization of soil C to CO_2, by contrast, is a broad process because the ability to mineralize and respire C substrates is relatively simple and shared by many microbial taxa (Schimel and Schaeffer, 2012). We thus expect that carbon mineralization would not respond strongly to changes in the composition of microbial communities.

Whether this framework of broad and narrow processes helps predict nutrient losses from agriculture depends on the relative importance of the multiple potential drivers of ecosystem process rates, including microbial community composition, nutrient availability, and soil and environmental properties. Though several studies have found support for microbial influence on narrow processes, such as denitrification, such studies often focus on identifying whether microbial community composition is related to ecosystem processes, but stop short of quantifying the relative contribution of the multiple controls on ecosystem processes (e.g., Philippot et al., 2013). Understanding the importance of biodiversity requires assessing the influence of composition relative to other biotic and abiotic controls (Laliberté and Tylianakis, 2012; Bradford et al., 2014).

Following theory (Schimel, 1995; Schimel and Schaeffer, 2012), we hypothesize that changes in microbial diversity will have a stronger effect on denitrification than will the direct effect of nutrient addition—measured as both N addition and the inclusion of seasonal legume rotations (henceforth *agroforestry*) to increase soil C—if changes in diversity correspond with changes in the relative abundance of denitrifying taxa and the abundances of functional genes involved in denitrification. Because C mineralization is a broad process, we expect that nutrient addition will have a stronger effect on process rates than changes in the microbial community.

MATERIALS AND METHODS

SITE SELECTION

We examine our hypotheses on 24 smallholder farms in western Kenya participating in the Millennium Villages Project (MVP) site in Sauri, Kenya (**Figure 1**; Wood et al., 2015). The center of the study area is located at 0°06′04.88 N, 34°30′40.12 E at an elevation of 1450 m. The mean annual temperature and precipitation for the study region are 24°C and 1800 mm, respectively. Annual precipitation is distributed bi-modally with 1120 mm in a long rainy season from March to June and 710 mm in a short rainy season from September to December. The soils are classified as Oxisols and are well drained sandy clay loams (on average 53.75% sand, 12.59% silt, 33.54% clay) with a mean pH of 5.45 and C:N of 11.52 (0–20 cm). The study zone was originally part of the moist broadleaf forest area in eastern and central Africa, but is now a mixed-maize agricultural system, with most farmers cultivating maize in both the long and short rainy seasons. Some farmers, however, replace the short rain maize crop with a seasonal legume rotation that fixes nitrogen and builds soil organic matter.

The MVP was designed to meet the Millennium Development Goals at the village scale in Sub-Saharan Africa and includes an agricultural component that focuses on increasing crop yields through mineral and organic nutrient addition to redress negative soil nutrient balances (Sanchez et al., 2007). This is primarily

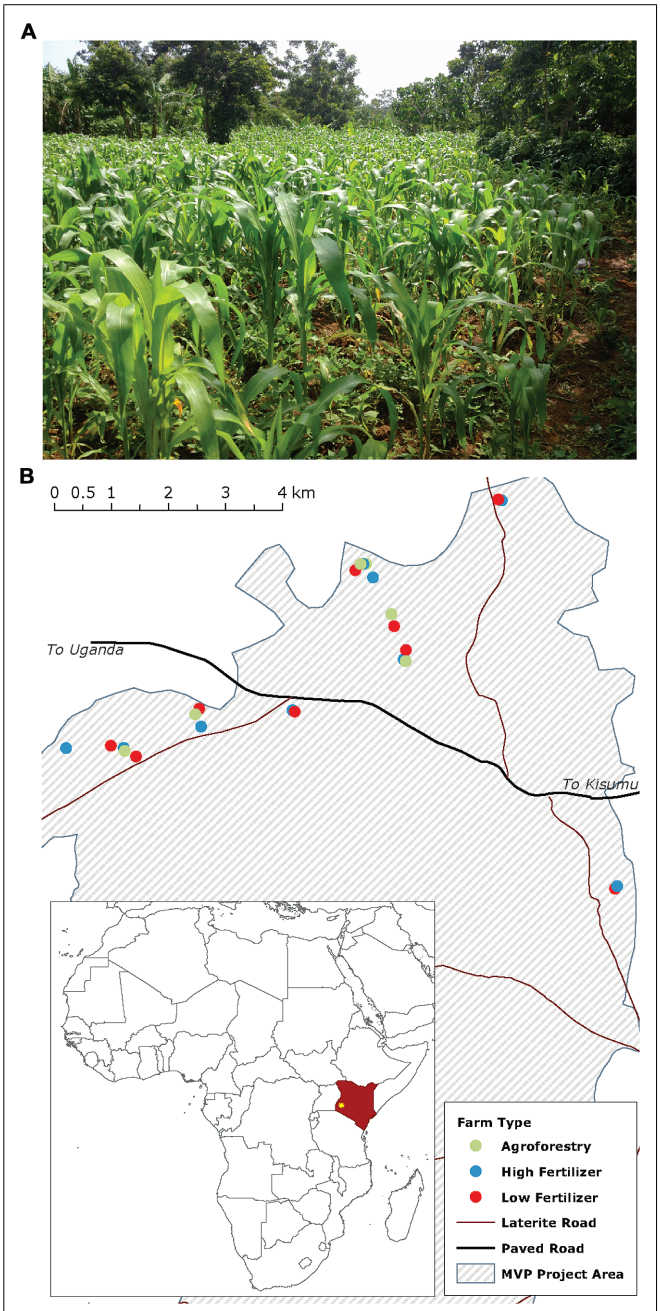

FIGURE 1 | Maize production in western Kenya mainly occurs on smallholder farms of around 1 hectare (A). Map **(B)** shows the study farms and their distribution across the Millennium Villages Project site area in western Kenya. Farm types are coded by color.

achieved by subsidizing mineral fertilizers (primarily diammonium phosphate and urea). Farmers are also trained in seasonal legume rotations to fix nitrogen and build soil organic matter. In Sauri, rotational legume trainings have been promoted since the early 1990s (Kiptot et al., 2007) and fertilizer subsidy programs were active from 2005 to 2008.

We selected farms to participate in the study based on 2 years of household surveys. We determined nutrient inputs and outputs for each of these farms through a combination of interviews, on-farm crop harvests, and biomass estimations. Farms were classified into three categories: *low fertilizer*, *high fertilizer*, and *high fertilizer + agroforestry* (specifically, seasonal legume rotations). Low fertilizer farms have applied less than 10 kg mineral N ha^{-1} y^{-1} since 2005; high fertilizer farms have applied at least 60 kg N ha^{-1} y^{-1} over the same time period. High fertilizer + agroforestry farms (henceforth *agroforestry*) apply amounts of mineral N comparable to *high fertilizer* farms, but also use agroforestry techniques to build soil organic matter. These agroforestry techniques replace short-rain maize crops with fast-growing leguminous tree, shrub, or herbaceous species that are planted from seed and cut each year for organic inputs to crop fields. These techniques are referred to generally as agroforestry, though agroforestry is a general term that captures different practices not studied here (e.g., wind breaks, live fencing, etc.). Our results, therefore, apply to agroforestry strategies that seasonally incorporate legume rotations.

We estimated the amount of N added to farms with farmer-reported data on the quantity of N added through mineral and organic sources (diammonium phosphate, urea, biological N_2-fixation, and manure). For agroforestry farms, we also estimated the amount of N added through N_2-fixation based on both literature-reported values and field-reported biomass estimates. To estimate the amount of N added through N_2-fixation we collected data on legume species planted, original planting density, thinning practices, wood harvesting, and legume management. We used plant density to estimate the amount of aboveground biomass N for each species present and then used literature data on the percent of total N derived from biological N_2-fixation for each species to calculate the amount of N derived from fixation (Gathumbi et al., 2002a,b; Ojiem et al., 2007). Because farmers tend to remove woody stems but incorporate fresh leaves, we removed the amount of N stored in woody biomass from this value to estimate the net N contribution from the legume species to the farm fields. We conservatively estimate that N_2-fixation contributed between 30 and 50 kg N ha^{-1} $year^{-1}$ during the short rain fallow, up to 30 kg of which may be due to the presence of *Mucuna pruriens*, an annual climbing legume (Ojiem et al., 2007). Planting densities, however, can vary widely from year-to-year with low-density years being as low as an order of magnitude less than those assumed in this estimate. Thus, depending on the year, actual fixation rates may be as low as 5–30 kg N ha^{-1} short rainy season^{-1}. We use the term 'nutrient addition' to refer to both N addition on low- and high-fertilizer and agroforestry farms as well as C addition through agroforestry. The final farms included in the study are distributed across the Sauri village cluster, but are clustered by treatment (**Figure 1**) on similar underlying soils.

SAMPLE COLLECTION AND MEASUREMENT

Soil sampling was conducted in June 2012, in the middle of the long rains, 2 weeks after fertilizer application. On the farm fields, we took 15 2-cm diameter soil cores from the top 20 cm of bulk soil. Cores were taken at regular intervals throughout the entire farm field and homogenized and aggregated to a composite sample. At each core location we recorded temperature and volumetric soil moisture content using a soil thermometer and a HydroSense moisture probe (Campbell Scientific, Logan, UT, USA). We sieved soils to 2 mm and stored soil for DNA extraction at −20° C. Soils for DNA extraction were transported to the U.S. within 1 week of sampling. Subsamples of sieved field soil were stored at 4° C, transported to the U.S. within 1 week of sampling, and used to determine pH, gravimetric soil moisture, and water holding capacity. Gravimetric soil moisture and water holding capacity (after wetting soils to field capacity) were determined by drying soil at 105°C for 24 h. Soil pH was determined using a benchtop meter of a 1:1 slurry of soil:H2O by volume.

A subsample of sieved soil was air-dried and used to determine total C and total N by combustion with an Elementar Vario Macro CNS analyzer. Total extractable P was assessed by combining a 5-g soil sample with 20 mL of Mehlich I extraction solution and shaking for 5 min followed by inductively coupled plasma spectrometry (Varian Vista MPX Radial ICP-OES). Soil nutrient assays were performed at the Auburn University Soil Testing Laboratory (AL, USA). Sieved, air-dried soil was also used to determine soil texture using the hydrometer method that uses sodium hexametaphosphate to complex the anions that bind to clay and silt particles into aggregates and suspend organic matter in solution. The density of the soil suspension is determined using a hydrometer after the sand particles settle and then after the silt particles settle (Bouyoucos method).

Denitrification and C mineralization assays were performed in Kenya on fresh soils at the MVP regional office in Kisumu, Kenya. Denitrification potential was estimated based on N_2O emissions during denitrifying enzyme activity (DEA) assays (Smith and Tiedje, 1979). In a 125-mL flask, we combined 20 g of soil with 20 mL of a 1-mM sucrose and 1-mM KNO_3^- solution. We fit each flask with a #5 stopper, which was inserted with a 22G needle capped with a stopcock. We then brought the headspace of the flask to 10% acetylene (C_2H_2) concentration by volume (to inhibit the reduction of N_2O to N_2 via denitrification). At the beginning of the incubation we closed the stopcocks and placed the flasks onto a shaker table at 125 rpm; flasks were only removed from the table for sampling. We sampled the headspace five times: at 30, 150, 210, and 270 min, by removing 30 mL of gas from the headspace and then replacing the volume of headspace that was removed with 30 mL of 10% C_2H_2 room air (fluxes were corrected for N_2O molecules removed at each sampling period). DEA headspace samples were stored in pre-evacuated vials.

Water-amended soil incubations were used to measure CO_2 efflux and, thus, actual C mineralization. These incubations were performed identically to the DEA incubations with three exceptions: (1) 20 mL of deionized water was added to soil in place of the sucrose and KNO_3^- solution; (2) no C_2H_2 was added to the headspace; and (3) headspace samples were taken

at only two time points (240 and 360 min). We also sampled room air at the beginning and end of each incubation and included travel standards to accompany samples, in order to correct for any sample loss during transport and storage. DEA and CO_2 headspace samples were transported to the U.S., where we determined N_2O and CO_2 concentrations by gas chromatography using a Shimadzu GC-14 GC with electron capture (for N_2O) and thermal conductivity (for CO_2) detectors at the Cary Institute (Millbrook, NY).

To measure taxonomic diversity, we performed 16S rRNA amplicon sequencing of bacteria and archaea following standard protocols of the Earth Microbiome Project using an Illumina MiSeq instrument ([1]Gilbert et al., 2010; Caporaso et al., 2012). Briefly, we extracted DNA using a MoBio PowerSoil 96-well extraction kit and we amplified the 16S rRNA V4 gene from bacterial and archaeal genomes using the primers 515F (forward) and 806R (reverse; Caporaso et al., 2012). The 16S rRNA gene is a well-conserved gene in bacteria and thus captures evolutionary relationships among bacterial taxa. Quality filtering was performed by comparing input sequences with Phred scores ($Q \geq 20$). Sequences shorter than 75% of the Phred score were discarded as well as sequences with ambiguous base call characters. All quality filtering and demultiplexing were performed using the split_libraries_fastq.py algorithm in QIIME and its associated default parameters ([1]Caporaso et al., 2010). Sequence reads were were binned into operational taxonomic units (OTUs) at a 97% similarity threshold. OTUs were then compared to GenBank to identify bacterial lineages. A total of 3,462,835 bacterial sequences were generated across all samples, representing 29,195 OTUs. Sequence lengths averaged 150.63 ± 2.93 per sample. Rarefaction was used to compare samples at depth of 40 sequences per sample. We calculated taxonomic diversity as Shannon diversity (H') of all OTUs. We calculated other diversity metrics, such as Faith's PD, and found similar patterns. All data checks and processing were done using QIIME (Caporaso et al., 2010).

To estimate microbial functional diversity, we measured the abundance of key functional genes using GeoChip 4.0 to analyze DNA samples that were extracted following the protocol for taxonomic assessment. GeoChip is a functional gene array of bacteria, archaea, and fungi covering 401 gene categories involved in major biogeochemical and ecological processes, as previously described (He et al., 2007; Yang et al., 2013; Tu et al., 2014). GeoChip examines the abundance of thousands of functional gene variants simultaneously through a fluorescent procedure. DNA samples were labeled with a fluorescent dye and purified using a QIA quick purification kit (Qiagen, Valencia, CA, USA) following He et al. (2007) and Tu et al. (2014). DNA was then dried in a SpeedVac (ThermoSavant, Milford, MA, USA) and labeled DNA was resuspended in a hybridization solution before hybridization of DNA was carried out on a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA). GeoChip microarrays were scanned by a NimbleGen MS200 scanner (Roche, Madison, WI, USA). Poor quality spots were removed when flagged as one or three by ImaGene (Arrayit, Sunnyvale, CA, USA) or with a signal-to-noise ratio of less than 2.0. Signal-to-noise ratio indicates the amount of luminescence from the sample compared to background noise. Average signal-to-noise ratios are often greater than 50 (He et al., 2007), so 2.0 represents high noise to signal. Processed data were subjected to the following steps: (i) normalize the signal intensity by dividing the signal intensity by the total intensity of the microarray followed by multiplying by a constant; (ii) transform by the natural logarithm; (iii) remove genes detected in only one out of three samples from the same treatment. Signal intensities were quantified and processed using a previously described data analysis procedure (He et al., 2007; Yang et al., 2013). We calculated functional diversity as Shannon diversity (H') of the signal intensity for all of the genes reported from the array. We also analyzed the response of individual denitrification genes to changes in functional diversity. These include genes involved in nitrite reduction (*nirK*, *nirS*), nitrate reduction (*narG*), and nitric oxide reduction (*norB*). GeoChip also includes *nosZ*, which is involved in nitrous oxide reduction, but we do not analyze this gene because it is involved in a later stage of denitrification than represented by the denitrification potential assay.

DATA ANALYSIS

We used structural equation models to simultaneously estimate each of the pathways among nutrient addition, soil and environmental properties (pH, texture, and moisture), microbial communities, and ecosystem processes while accounting for correlations between multiple response variables (Grace, 2006). Structural equation modeling is increasing used in ecology and environmental sciences to assess the relative impacts of multiple variables on each other and a set of response variables (Grace, 2006). This technique has been applied to a wide range of issues in ecology and environmental sciences (e.g., Byrnes et al., 2011; Flynn et al., 2011; Laliberté and Tylianakis, 2012). Relevant to our study, it was used by Colman and Schimel (2013) to determine the drivers of microbial respiration and N mineralization at continental scales.

To test our hypotheses about the relative importance of nutrient addition and microbial composition, we first fitted models including both nutrient addition and microbial diversity variables. Soil pH was the only significant environmental control and was thus the only environmental variable retained in the final models. We then fitted models to optimize goodness-of-fit and do not include variables that do not contribute strongly to model goodness-of-fit. Different models were fitted for each of the two response variables (denitrification potential and C mineralization). For each response variable, constrained (microbial + nutrient addition) and unconstrained models were compared based on change in AIC values. The final, unconstrained model retained nutrient addition and pH, but did not include microbial diversity.

We report standardized path estimates that allow for comparison of the relative magnitude of variables within the same model (Grace and Bollen, 2005). For model goodness-of-fit, we report X^2 and root mean square error of approximation (RMSEA). These measures assess the similarity between the covariance matrix of the observed data and the covariance matrix implied by the specified model. A X^2 P-value greater than 0.05 implies significant

[1]http://www.earthmicrobiome.org/emp-standard-protocols/

Table 1 | Means and SD for variables included in structural equation models among the three categories of nutrient addition: low fertilizer, high fertilizer, and agroforestry.

Farm type	Denitrification (*ng N g dry soil^{-1} h^{-1}*)	C mineralization (*ug C g dry soil^{-1} h^{-1}*)	Taxonomic diversity	Functional diversity	Sand	Silt	Clay	pH	C	N	P
			H′			*%*		*log [H$^+$]*	*%*	*%*	*ppm*
Low fertilizer	0.61	1.04	10.02	8.88	53.76	14.40	31.74	5.41	1.83	0.20	16.63
	[0.49]	[0.24]	[0.31]	[0.07]	[5.64]	[7.61]	[6.34]	[0.35]	[0.20]	[0.03]	[9.15]
High fertilizer	0.48	0.99	9.78	8.99	56.00	9.71	34.15	5.06	1.95	0.22	19.13
	[0.09]	[0.41]	[0.45]	[0.08]	[3.13]	[5.91]	[6.57]	[0.37]	[0.16]	[0.03]	[10.30]
Agroforestry	1.00	1.27	9.79	9.05	58.58	10.46	30.86	5.47	1.72	0.18	7.00
	[0.58]	[0.13]	[0.30]	[0.09]	[2.06]	[4.67]	[4.96]	[0.72]	[0.27]	[0.02]	[2.55]

All soil properties are to a depth of 20 cm. Because of unbalanced design statistical comparisons between groups are not valid; instead the effect of Farm type is represented by the path coefficients of Agroforestry and N Addition in the structural equation models. Further detail on changes in soil properties is presented in Wood et al. (2015).

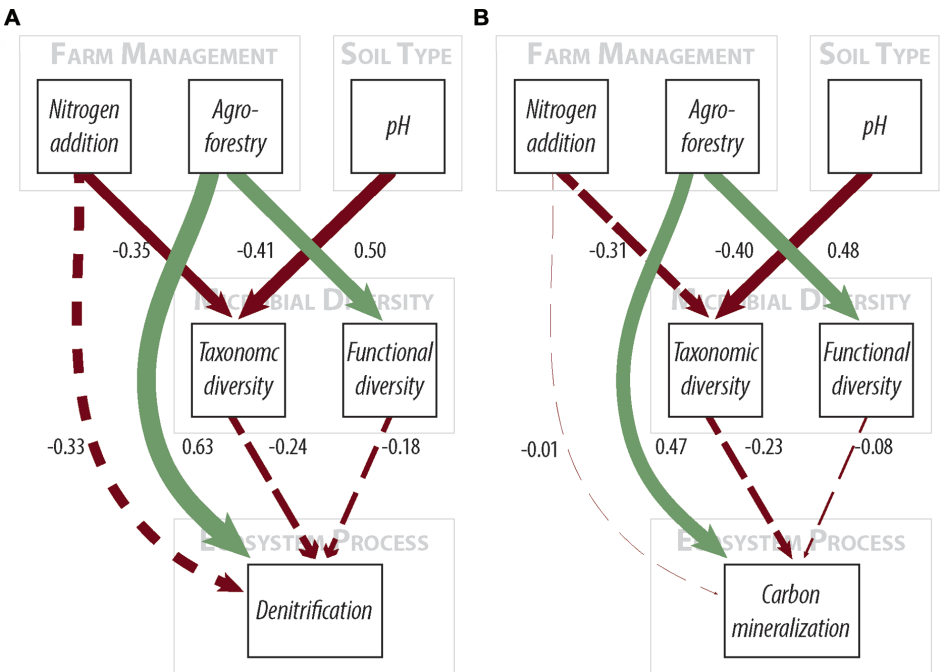

FIGURE 2 | Path diagrams for structural equation models of the relationship between farm management, microbial diversity, and (A) denitrification enzyme activity or (B) carbon mineralization. Models **(A,B)** show the relative effect of management and microbial diversity. Solid paths are statistically significant at $p < 0.10$. Dashed paths are insignificant, but were included for hypothesis testing or overall model fit. Line color represents effect direction (light green = positive, deep red = negative). Path widths are proportional to standardized regression coefficients, which are shown next to each path. Results and model statistics are in **Table 2**.

overlap between the observed and implied data, and thus adequate model fit. We report Sartorra-Bentler X^2 correction factors to improve estimates based on violations of multivariate normality. Because the X^2 test is based on large sample theory, we also report RMSEA, which is a goodness-of-fit measure weighted by sample size. We use an RMSEA value below 0.1 to represent good model fit because for sample sizes less than 50, the conventional RMSEA cut-off value of 0.05 is overly conservative (Chen et al., 2008). Individual paths were estimated using maximum likelihood and we considered paths to be significant at $P < 0.05$ and marginally significant at $P < 0.10$ (Hurlbert and Lombardi, 2009). Insignificant paths were excluded from models unless they significantly improved overall model fit, based on X^2 and RMSEA values as well as assessment of modification indices (Grace, 2006). All models were fitted using the *lavaan* package in R (Rosseel, 2012).

Table 2 | Model results and goodness of fit statistics for structural equation models.

Denitrification		
	Standardized estimate	***P***
Denitrification~		
Agroforestry	0.63	0.00
Functional diversity	−0.18	0.31
N addition	−0.33	0.10
Taxonomic diversity	−0.24	0.18
Taxonomic diversity~		
N Addition	−0.35	0.06
pH	−0.41	0.00
Functional diversity~		
Agroforestry	0.50	0.01
Structural equation model metrics		
n	21	
df	5	
χ^2	2.14	
$P_{\chi 2}$	0.83	
RMSEA	0.00	
P_{RMSEA}	0.85	

C Mineralization		
	Standardized estimate	***P***
C mineralization~		
Agroforestry	0.47	0.00
Functional diversity	−0.08	0.72
N addition	−0.01	0.95
Taxonomic diversity	−0.23	0.35
Taxonomic diversity~		
N Addition	−0.31	0.18
pH	−0.40	0.01
Functional diversity~		
Agroforestry	0.48	0.03
Structural equation model metrics		
n	21	
df	5	
χ^2	2.62	
$P_{\chi 2}$	0.76	
RMSEA	0.00	
P_{RMSEA}	0.75	

We report robust X^2 statistics for model fit. $P > 0.05$ indicates that estimated models have covariance matrices among variables that are not strongly different from observed values and that the model, therefore, adequately represents the data. Root mean square error of approximation (RMSEA) is a sample-size weighted measure of model fit. Values below 0.1 indicate good model fit.

RESULTS

We hypothesized that changes in microbial diversity would have a stronger effect on denitrification than would the direct effect of nutrient addition if changes in diversity correspond with changes in the relative abundance of denitrifying taxa and/or the abundance of associated genes involved in denitrification. We also hypothesized that nutrient addition would be a stronger predictor of C mineralization, a broad process, than microbial diversity.

We find that farm management—through N addition and agroforestry—impacts the taxonomic and functional diversity of soil microbial communities. Specifically, taxonomic diversity decreases by 2.40% from low-to-high N addition (**Table 1**), though this effect is weaker than the effect of pH, which is also associated with lower taxonomic diversity (**Figures 2A,B**). We did not find that these changes in taxonomic diversity were coupled with changes in the relative abundance of select groups of denitrifying taxa (**Figure 3**). Agroforestry was the strongest driver of functional diversity, which increased 1% between high fertilizer and agroforestry farms and 2% between low fertilizer and agroforestry farms (**Table 1**; **Figures 2A,B**). We did find that greater functional diversity is significantly related to greater abundances of several genes involved in denitrification: *nirK*, *nirS*, *norB*, and *narG* (**Figure 4**).

We did not, however, find that changes in taxonomic and functional diversity were related to rates of either denitrification or C mineralization. Instead, ecosystem process rates were most strongly linked to the direct effect of farm management. Denitrification decreased by 21.31% from low-to-high N and increased by 63.93% from low N to agroforestry (**Table 1**). The path estimate for agroforestry on denitrification (0.63) is three times greater than the coefficient for either taxonomic diversity (−0.24) or functional diversity (−0.18). The agroforestry coefficient is also twice the magnitude of the coefficient for N addition (−0.33). We find support for our hypothesis that C mineralization will be more influenced by nutrient addition than microbial community composition. C mineralization rates were 4.81% lower on high-vs.-low N farms and 22.12% greater under agroforestry (**Table 1**). The path coefficient for the effect of agroforestry on C mineralization (0.47) is more than twice as great as the coefficient for taxonomic diversity (−0.23) and N addition (0.16) and around five times the effect of functional diversity (−0.08).

DISCUSSION

Our results reveal that shifts in microbial taxonomic and functional diversity due to farm management are not significantly related to either denitrification or C mineralization on smallholder farms in western Kenya. This finding supports our hypothesis that C mineralization would not be sensitive to changes in microbial communities because it is a broad process that can be carried out by many microbial taxa. However, we did not find support for our hypothesis that denitrification would be sensitive to community

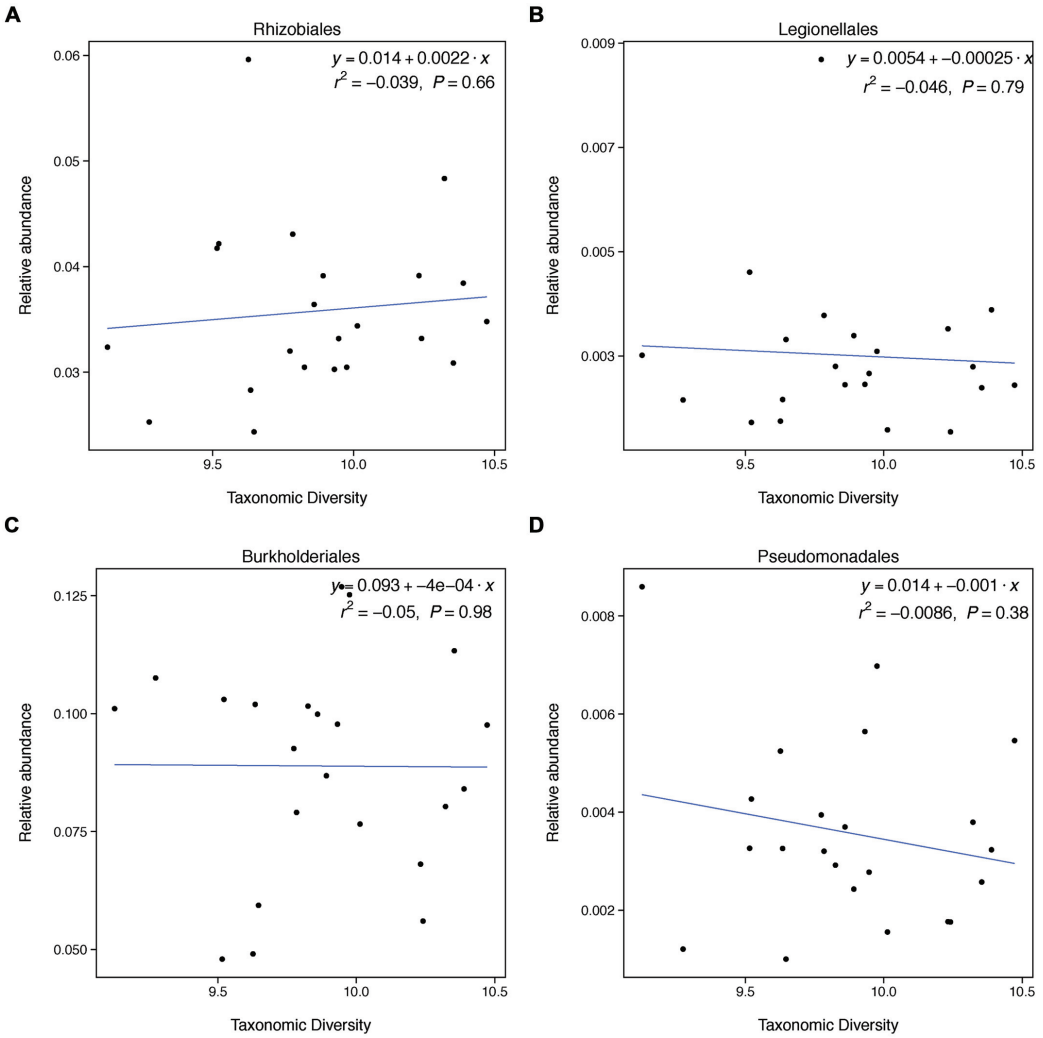

FIGURE 3 | Taxonomic diversity is not related to changes in the relative abundances of select denitrifying taxa. These groups do not represent all categories of denitrifying taxa and not all taxa within these categories are able to carry out denitrification. These groups were selected because they broadly represent evolutionary lineages that are capable of denitrification and had relatively high relative abundances in our samples.

change because it is a narrow process carried out by relatively few taxa.

This unexpected result may be explained by the fact that changes in taxonomic diversity were not coupled with decreases in the relative abundance of denitrifying taxa. Our hypothesis was built on the expectation that diversity would relate to denitrification rates if changes in diversity were paired with changes in the relative abundance of taxa able to carry out denitrification. Because denitrifying taxa are found widely through the microbial phylogeny, it is difficult to identify groups of taxa that are all denitrifiers. However, we found that groups that broadly contain denitrifiers do not change in relative abundance with changes in diversity. This finding may explain why taxonomic diversity was not a significant predictor of denitrification.

We also expected that functional diversity would be a significant control on denitrification if changes in functional diversity were coupled with changes in the abundances of key denitrifying genes. We did find a strong coupling between our functional diversity metric (Shannon diversity of all functional genes from GeoChip) and the abundances of four particular genes key to denitrification: *nirK*, *nirS*, *narG*, and *norB*. Thus, our finding that functional diversity was not significantly related to rates of denitrification was unexpected. However, the finding fits with recent meta-analysis showing that microbial functional gene abundances are rarely strongly correlated with corresponding process rates (Rocca et al., 2014). Our lack of observed relationship between gene abundances and process rates may be explained by the fact that our measure of functional diversity is based on the presence of functional genes using DNA. Because DNA only indicates the presence of a gene, rather than whether that gene is expressed, our measure of functional diversity only represents a coarse picture of microbial functional capacity. Our results thus suggest that rates of denitrification are more strongly controlled by the expression of

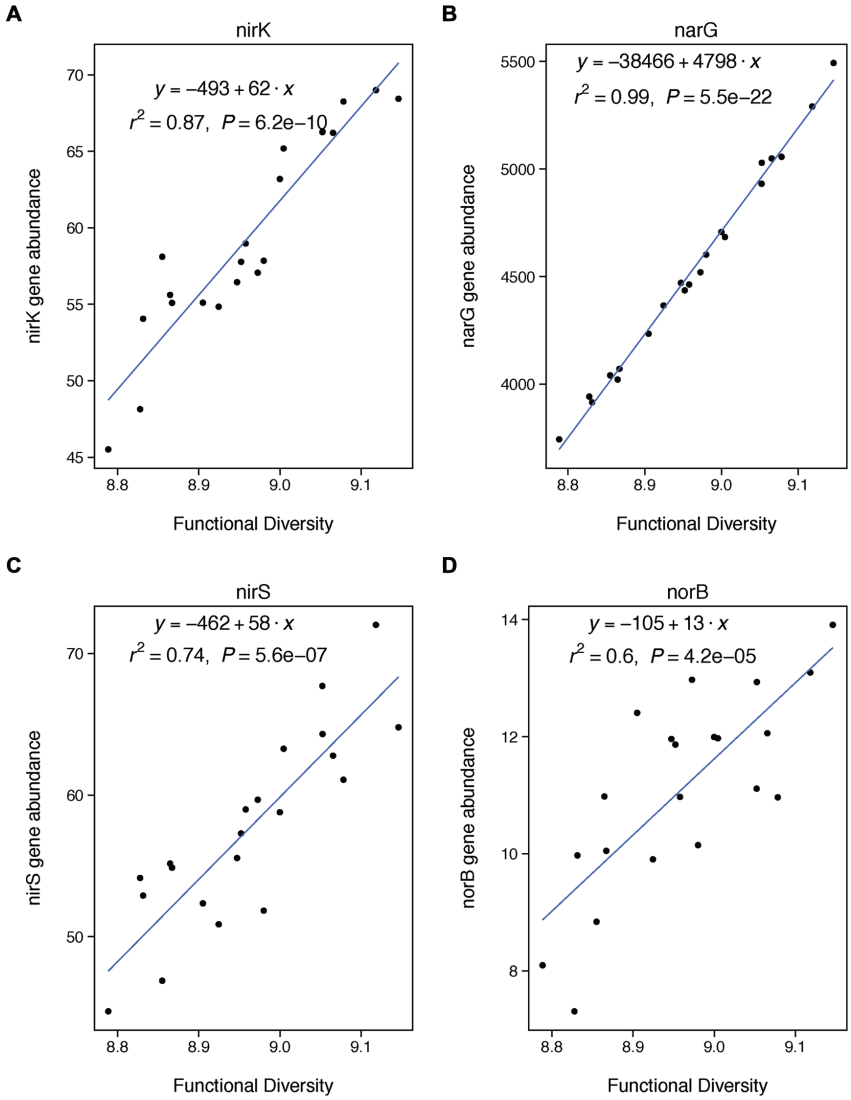

FIGURE 4 | Functional diversity is positively correlated with changes in the abundances of specific genes involved in denitrification. These genes are involved in nitrite reduction (**A**: nirK, **C**: nirS), nitrate reduction (**B**: narG), and nitric oxide reduction (**D**: norB). We did not analyze nosZ because it is involved in a later stage of denitrification than included in our potential assay (nitrous oxide reduction).

functional genes, rather than their overall abundance. This finding suggests that coarse measures of microbial communities based on DNA—whether taxonomic or functional—may be insufficient to understanding the changes in the functional contributions of these communities under certain types of land management (Rocca et al., 2014).

Though understanding when microbial communities should impact ecosystem process rates is well established, we show that actual changes in microbial communities observed in a tropical agroecosystem are not a strong predictor of denitrification rates because changes in microbial communities are relatively minor in magnitude. Our findings, however, do not invalidate the hypothesis that narrow processes are sensitive to community composition and broad processes are not, which has been supported in previous work (e.g., Salles et al., 2012; Schimel and Schaeffer, 2012; Philippot et al., 2013; Powell et al., 2015). Instead, our findings raise doubts about the relative importance of microbial community composition compared to direct effects of nutrient addition on nutrient losses in agricultural settings. This is because the magnitude of change in microbial diversity induced by land management was not large enough to significantly impact ecosystem process rates. As a result, the direct effect of farm management (rather than the indirect effect through changes in microbial communities) was the dominant control of both denitrification and C mineralization. Whether changes in microbial community composition translate into changes in rates of ecosystem processes controlled by soil microbes is of great interest in soil ecology (e.g., Torsvik and Øvreås, 2002; Philippot and Hallin, 2005; van der Heijden et al., 2008), but remains an ongoing debate (Schimel and Schaeffer, 2012). Our study is unique, however, in

that few studies have connected changes in microbial communities to ecosystem process rates in a framework that assesses the relative importance of the multiple drivers of these ecosystem processes.

Although we focus on smallholder farms in western Kenya, there is a widespread effort to increase crop yields across sub-Saharan Africa and in tropical smallholder agriculture more generally (Wiggins et al., 2010). Because 75% of the world's 1.2 billion poorest people are engaged in smallholder, making up 500 million farms of less than 2 ha (Wiggins et al., 2010), our findings may help inform understanding of drivers of nutrient loss in tropical smallholder agriculture due to increased attention from international development organizations.

It is becoming widely recognized that agricultural sustainability requires agricultural practices that maximize multiple ecosystem services while minimizing nutrient losses to the environment (Foley et al., 2011; Bommarco et al., 2013). This is particularly important in tropical ecosystems that are undergoing large-scale modifications of nutrient cycling pathways due to intense efforts by the international development community to increase fertilizer use by tropical smallholder farmers. Further work should focus on understanding how management-induced shifts in microbial communities impact not just potential nutrient loss, but the multiple ecosystem services provided by soil and how such understanding can be integrated into sustainable agricultural strategies.

AUTHOR CONTRIBUTIONS

SAW and MA conceived research and performed lab and field work; SAW, MA, MAB, KLM, SN, CN, CAP, and KLT designed the study; JZ performed GeoChip analyses; SAW analyzed data and wrote the first draft of the manuscript; all authors contributed to interpretation of results and commented on the manuscript. The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00090/abstract

REFERENCES

Bommarco, R., Kleijn, D., and Potts, S. G. (2013). Ecological intensification: harnessing ecosystem services for food security. *Trends Ecol. Evol.* 28, 230–238. doi: 10.1016/j.tree.2012.10.012

Bradford, M. A., Wood, S. A., Bardgett, R. D., Black, H. I. J., Bonkowski, M., Eggers, T., et al. (2014). Discontinuity in the responses of ecosystem processes and multifunctionality to altered soil community composition. *Proc. Natl. Acad. Sci. U.S.A.* 111, 14478–14483. doi: 10.1073/pnas.1413707111

Byrnes, J. E., Reed, D. C., Cardinale, B. J., Cavanaugh, K. C., Holbrook, S. J., and Schmitt, R. J. (2011). Climate-driven increases in storm frequency simplify kelp forest food webs. *Glob. Chang. Biol.* 17, 2513–2524. doi: 10.1111/j.1365-2486.2011.02409.x

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platorms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Chen, F., Curran, P. J., Bollen, K. A., Kirby, J., and Paxton, P. (2008). An empirical evaluation of the use of fixed cutoff points in RMSEA test statistic in structural equation models. *Sociol. Methods Res.* 36, 462–494. doi: 10.1177/0049124108314720

Colman, B. P., and Schimel, J. P. (2013). Drivers of microbial respiration and net N mineralization at the continental scale. *Soil Biol. Biochem.* 60, 65–76. doi: 10.1016/j.soilbio.2013.01.003

Fierer, N., Lauber, C. L., Ramirez, K. S., Zaneveld, J., Bradford, M. A., and Knight, R. (2011). Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *ISME J.* 6, 1007–1017. doi: 10.1038/ismej.2011.159

Flynn, D. F. B., Mirotchnik, N., Jain, M., Palmer, M. I., and Naeem, S. (2011). Functional and phylogenetic diversity as predictors of biodiversity-ecosystem-function relationships. *Ecology* 92, 1573–1581. doi: 10.1890/10-1245.1

Foley, J. A., Ramankutty, N., Brauman, K. A., Cassidy, E. S., Gerber, J. S., Johnston, M., et al. (2011). Solutions for a cultivated planet. *Nature* 478, 337–342. doi: 10.1038/nature10452

Gathumbi, S. M., Cadisch, G., and Giller, K. E. (2002a). 15N natural abundance as a tool for assessing N2-fixation of herbaceous, shrub and tree legumes in improved fallows. *Soil Biol. Biochem.* 34, 1–13. doi: 10.1016/S0038-0717(02)00038-X

Gathumbi, S. M., Ndufa, J. K., Giller, K. E., and Cadisch, G. (2002b). Do species mixtures increase above- and belowground resource capture in woody and herbaceous tropical legumes? *Agron. J.* 94, 518–526. doi: 10.2134/agronj2002.5180

Gilbert, J. A., Meyer, F., Jansson, J., Gordon, J., Pace, N., Tiedje, J., et al. (2010). The earth microbiome project: meeting report of the "1st EMP meeting on sample selection and acquisition" at Argonne National Laboratory October 6th 2010. *Stand. Genomic Sci.* 3, 249–253. doi: 10.4056/aigs.1443528

Grace, J. B. (2006). *Structural Equation Modeling and Natural Systems.* New York: Cambridge University Press. doi: 10.1017/CBO9780511617799

Grace, J. B., and Bollen, K. A. (2005). Interpreting the results from multiple regression and structural equation models. *Bull. Ecol. Soc. Am.* 86, 283–295. doi: 10.1890/0012-9623(2005)86[283:ITRFMR]2.0.CO;2

He, Z., Gentry, T. J., Schadt, C. W., Wu, L., Liebich, J., Chong, S. C., et al. (2007). GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME J.* 1, 67–77. doi: 10.1038/ismej.2007.2

Hurlbert, S. H., and Lombardi, C. M. (2009). Final collapse of the Neyman-Pearson decision theoretic framework and rise of the neoFisherian. *Ann. Zool. Fenn.* 46, 311–349. doi: 10.5735/086.046.0501

Kiptot, E., Hebinck, P., Franzel, S., and Richards, P. (2007). Adopters, testers or pseudo-adopters? Dynamics of the use of improved tree fallows by farmers in western Kenya. *Agric. Syst.* 94, 509–519. doi: 10.1016/j.agsy.2007.01.002

Krause, S., Le Roux, X., Niklaus, P. A., Van Bodegom, P. M., Lennon, J. T., Bertilsson, S., et al. (2014). Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. *Front. Microbiol.* 5:251. doi: 10.3389/fmicb.2014.00251

Laliberté, E., and Tylianakis, J. M. (2012). Cascading effects of long-term land-use change on plant traits and ecosystem functioning. *Ecology* 93, 145–155. doi: 10.1890/11-0338.1

Ojiem, J. O., Vanlauwe, B., de Ridder, N., and Giller, K. E. (2007). Niche-based assessment of contributions of legumes to the nitrogen economy of Western

Kenya smallholder farms. *Plant Soil* 292, 119–135. doi: 10.1007/s11104-007-9207-7

Pett-Ridge, J., and Firestone, M. (2005). Redox fluctuation structures microbial communities in a wet tropical soil. *Appl. Environ. Microbiol.* 71, 6998–7007. doi: 10.1128/AEM.71.11.6998-7007.2005

Philippot, L., and Hallin, S. (2005). Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. *Curr. Opin. Microbiol.* 8, 234–239. doi: 10.1016/j.mib.2005.04.003

Philippot, L., Spor, A., Hénault, C., Bru, D., Bizouard, F., Jones, C. M., et al. (2013). Loss in microbial diversity affects nitrogen cycling in soil. *ISME J.* 7, 1609–1619. doi: 10.1038/ismej.2013.34

Powell, J. R., Welsh, A., and Hallin, S. (2015). Microbial functional diversity enhances predictive models linking environmental parameters to ecosystem properties. *Ecology* doi: 10.1890/14-1127.1 [Epub ahead of print].

Ramirez, K. S., Craine, J. M., and Fierer, N. (2012). Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Glob. Change Biol.* 18, 1918–1927. doi: 10.1111/j.1365-2486.2012.02639.x

Ramirez, K. S., Lauber, C. L., Knight, R., Bradford, M. A., and Fierer, N. (2010). Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. *Ecology* 91, 3463–3470. doi: 10.1890/10-0426.1

Rocca, J. D., Hall, E. K., Lennon, J. T., Evans, S. E., Waldrop, M. P., Cotner, J. B., et al. (2014). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. *ISME J.* doi: 10.1038/ismej.2014.252 [Epub ahead of print].

Rosseel, Y. (2012). Lavaan: an R package for structural equation modeling. *J. Stat. Softw.* 48, 1–36.

Salles, J. F., Le Roux, X., and Poly, F. (2012). Relating phylogenetic and functional diversity among denitrifiers and quantifying their capacity to predict community functioning. *Front. Microbiol.* 3:209. doi: 10.3389/fmicb.2012.00209

Sanchez, P., Palm, C. A., Sachs, J., Denning, G., Flor, R., Harawa, R., et al. (2007). The African millennium villages. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16775–16780. doi: 10.1073/pnas.0700423104

Schimel, J. (1995). "Ecosystem consequences of microbial diversity and community structure," in *Arctic and Alpine Biodiversity: Patterns, Causes and Ecosystem Consequences*, eds F. S. Chapin and C. Körner (Berlin: Springer), 239–254. doi: 10.1007/978-3-642-78966-3_17

Schimel, J., and Schaeffer, S. (2012). Microbial control over carbon cycling in soil. *Front. Microbiol.* 3:348. doi: 10.3389/fmicb.2012.00348

Smith, M. S., and Tiedje, J. M. (1979). Phases of denitrification following oxygen depletion in soil. *Soil Biol. Biochem.* 11, 261–267. doi: 10.1016/0038-0717(79)90071-3

Torsvik, V., and Øvreås, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5, 240–245. doi: 10.1016/S1369-5274(02)00324-7

Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., van Nostrand, J. D., et al. (2014). GeoChip 4: a functional gene array-based high throughput environmental technology for microbial community analysis. *Mol. Ecol. Res.* 14, 914–928.

van der Heijden, M. G. A., Bardgett, R. D., and Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol. Lett.* 11, 296–310. doi: 10.1111/j.1461-0248.2007.01139.x

van der Heijden, M. G. A., and Wagg, C. (2013). Soil microbial diversity and agro-ecosystem functioning. *Plant Soil* 363, 1–5. doi: 10.1007/s11104-012-1545-4

Vitousek, P., Naylor, R., Crews, T., David, M. B., Drinkwater, L. E., Holland, E., et al. (2009). Nutrient imbalances in agricultural development. *Science* 324, 1519–1520. doi: 10.1126/science.1170261

Wallenstein, M. D., and Hall, E. K. (2012). A trait-based framework for predicting when and where microbial adaptation to climate change will affect ecosystem functioning. *Biogeochemistry* 109, 35–47. doi: 10.1007/s10533-011-9641-8

Wessén, E., Söderström, M., Stenberg, M., Bru, D., Hellman, M., Welsh, A., et al. (2011). Spatial distribution of ammonia-oxidizing bacteria and archaea across a 44-hectare farm related to ecosystem functioning. *ISME J.* 5, 1213–1225. doi: 10.1038/ismej.2010.206

Wiggins, S., Kirsten, J., and Llambí, L. (2010). The future of small farms. *World Dev.* 38, 1341–1348. doi: 10.1016/j.worlddev.2009.06.013

Wood, S. A., Bradford, M. A., Gilbert, J. A., McGuire, K. L., Palm, C. A., Tully, K. L., et al. (2015). Agricultural intensification and the functional capacity of soil microbes on smallholder African farms. *J. Appl. Ecol.* (in press). doi: 10.1111/1365-2664.12416

Yang, Y., Wu, L., Lin, Q., Yuan, M., Xu, D., Yu, H., et al. (2013). Responses of the functional structure of soil microbial community to livestock grazing in the Tibetan alpine grassland. *Glob. Change Biol.* 19, 637–648. doi: 10.1111/gcb.12065

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Comparison of Fungal Community in Black Pepper-Vanilla and Vanilla Monoculture Systems Associated with Vanilla *Fusarium* Wilt Disease

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Long-term vanilla monocropping often results in the occurrence of vanilla *Fusarium* wilt disease, seriously affecting its production all over the world. In the present study, vanilla exhibited significantly less *Fusarium* wilt disease in the soil of a long-term continuously cropped black pepper orchard. The entire fungal communities of bulk and rhizosphere soils between the black pepper-vanilla system (i.e., vanilla cropped in the soil of a continuously cropped black pepper orchard) and vanilla monoculture system were compared through the deep pyrosequencing. The results showed that the black pepper-vanilla system revealed a significantly higher fungal diversity than the vanilla monoculture system in both bulk and rhizosphere soils. The UniFrac-weighted PCoA analysis revealed significant differences in bulk soil fungal community structures between the two cropping systems, and fungal community structures were seriously affected by the vanilla root system. In summary, the black pepper-vanilla system harbored a lower abundance of *Fusarium oxysporum* in the vanilla rhizosphere soil and increased the putatively plant-beneficial fungal groups such as *Trichoderma* and *Penicillium* genus, which could explain the healthy growth of vanilla in the soil of the long-term continuously cropped black pepper field. Thus, cropping vanilla in the soil of continuously cropped black pepper fields for maintaining the vanilla industry is executable and meaningful as an agro-ecological system.

Keywords: continuous cropping, black pepper, soil fungal communities, Miseq sequencing, vanilla healthy growth

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INTRODUCTION

Vanilla (*Vanilla planifolia*), a herbaceous perennial vine with high economic value, has been widely cropped in tropical and subtropical regions (Minoo et al., 2008). However, the long-term monoculture of this crop often results in the occurrence of soil-borne *Fusarium* wilt disease, seriously affecting its production worldwide in vanilla-cropping regions (Jayasekhar et al., 2008; Pinaria et al., 2010; Xiong et al., 2015b) and leading to significant economic losses over the last decade. Fungicides and biological control agents have been traditionally suggested as integrated control strategies for vanilla *Fusarium* wilt disease (Tombe and Sitepu, 1986; Sandheep et al., 2012); however, these methods are usually environmentally unfriendly or inefficient. Thus, exploring an effective method for controlling the vanilla *Fusarium* wilt disease is extremely important for

maintaining the vanilla production. Meanwhile, in tropical China, multiple continuous cropping fields for other tropical crops, such as black pepper and banana, have suffered serious successive cropping obstacles and were given up for growing the same crops (Wang et al., 2013; Xiong et al., 2015a). Because of these associated problems, farmers naturally grow different crops in these fields. By some chance, after our field investigation, an interesting phenomenon was always observed where vanilla with the lowest *Fusarium* wilt disease incidence (DI) grew in the continuously cropped black pepper field. The causes of the disease decline might be very complex, such as improved soil physical and chemical properties and land management practices (Hilton et al., 2013; Navarro-Noya et al., 2013). However, the detailed mechanisms involved in the healthy vanilla growth associated with the soil microflora variation under the soil of long-term continuously cropped black pepper fields remain unclear.

Exploring continuously cropped field soil for other crop growth is meaningful and sustainable to agro-ecological systems. Meanwhile, to our limited knowledge, few studies have focused on the long-term continuously cropped soil supporting other crop growth; thus, how the variation in soil microbiota under long-term continuously cropped soil could support other crop growth is even less well understood. Soil microorganisms play critical roles in regulating soil fertility, global nutrient cycling, and plant health (Fierer et al., 2012), which might be directly linked to the maintenance of plant health in agro-systems. Within soil ecosystems, the immediate surroundings of the plant root, i.e., the rhizosphere, is a dynamic interface supporting the exchange of resources between plants and their associated soil environment (Peiffer et al., 2013). Rhizosphere microbiota, considered as the second genome of the plant, are significantly influenced by plant roots (Philippot et al., 2013). The main source of microbial communities in the rhizosphere is the adjacent root-free soil, called the bulk soil; hence, the changes brought about in the communities of the bulk soil will have an effect on the assembly and the final composition of rhizosphere communities (Mendes et al., 2014).

The development of high-throughput sequencing, particularly Illumina MiSeq sequencing (Metzker, 2010; Shokralla et al., 2012), offers a powerful strategy for uncovering the complex and diverse soil microbial communities with high throughput, high accuracy, and considerably lower cost. The internal transcribed spacers (ITS1) region has been widely used in the analysis of soil fungal communities (Xu et al., 2012; Lu et al., 2013). The functional diversity of soil fungi and their capacity to colonize diverse microhabitats can influence pathogen levels and play a significant role in improving plant health (Penton et al., 2014). Given that vanilla *Fusarium* wilt disease is caused by a fungal pathogen, exploring the fungal community involved in the healthy growth of vanilla in black pepper-vanilla agro-ecosystems is quite important.

Thus, in this study, we hypothesize that long-term continuous cropping black pepper orchards harbored a unique soil fungal community associated with healthy vanilla growth. To test this hypothesis, we used pot experiments to evaluate the persistent ability of the soil of long-term continuously cropped black pepper fields to support vanilla healthy growth; and fungal community of the bulk and rhizosphere soils in the black pepper-vanilla and vanilla monoculture systems was accessed by the Illumina MiSeq sequencing.

MATERIALS AND METHODS

No specific permits were required for the described field studies. The locations are not protected. The field studied did not involve endangered or protected species.

Experiment Descriptions

The experimental site is located at the Spice and Beverage Research Institute, Wanning City, Hainan Province, China (110°19′E-110°22′E, 18°72′N-18°76′N). It is an area with a tropical monsoon climate, a mean annual temperature of 24.5°C and a mean annual precipitation of 2201 mm. The experimental soil was collected in April 2013 from the 20-years continuously cropped black pepper orchard. The soil was mixed thoroughly and transferred to the greenhouse with an average temperature of 30°C and an average humidity of 72% at the Spice and Beverage Research Institute. Meanwhile, the soil ∼200 m away from the black pepper orchard collected from the 21-year continuously cropped vanilla orchard showing serious vanilla *Fusarium* wilt disease (Xiong et al., 2015b) was considered a control. Both the black pepper and vanilla orchard soils are sandy loam in texture and developed from the same parent material. The experiment was performed using a randomized complete block design in three replicates, where each block had six pots for each treatment, and each pot contained 15 kg soil with three seedling vanillas. The agronomic management and fertilization regime were uniform during the next 18 months (April, 2013 to October, 2014). Vanilla *Fusarium* wilt disease was monitored immediately after the seedlings were transplanted into the pots based on the observation of typical wilt symptoms. The DI was calculated as the percentage of infected plants among the total number of plants (Wei et al., 2011). It is worth noting that we also used continuously cropped banana and coffee orchards soil to cultivate vanilla in pots. We found the continuously cropped black pepper soil showed the lowest vanilla *Fusarium* wilt disease and the highest plant biomass (vanilla shoot dry weight). Hence, we got the two vanilla cropping regimes, i.e., black pepper-vanilla system and vanilla monoculture system for the subsequent research.

Soil Sample Collection and DNA Extraction

After removing the vanilla plants from the pots, the bulk soil samples obtained for each replicate from the black pepper-vanilla and vanilla monoculture systems were referred to as "BB" and "VB," respectively. All six bulk soil samples were passed through a 2 mm sieve, thoroughly homogenized and divided into 2 subsamples: one was air-dried for a soil characteristic analysis according to our previous methods (Xiong et al., 2015b), and the remainder was stored at −80°C for DNA extraction. For the vanilla rhizosphere soil samples, six vanilla plants were randomly selected from each replicate in the black pepper-vanilla and vanilla monoculture systems, the roots were vigorously shaken to dislodge the loosely adhering soil, and the soil remaining attached

to the root system was considered to be rhizosphere soil. The rhizosphere soil was collected using the following protocol: the roots were cut into pieces of ∼1 cm length and carefully mixed, 20 g of roots were pooled into a 500 mL vol. flask containing 200 mL ddH_2O and washed on a shaking platform for 20 min at 180 rpm, the washing buffer was subjected to centrifugation (10,000 g, 10 min), and then the resulting pellet was obtained and defined as the rhizosphere soil. The rhizosphere soil from the black pepper-vanilla system and vanilla monoculture system are referred to as "BR" and "VR," respectively. All six rhizosphere soil samples were stored at −80°C for DNA extraction.

Total DNA was extracted from the 12 soil samples using a MoBioPowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The genomic DNA concentration and purity were measured using NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE) spectrophotometry.

PCR Amplification and Deep Pyrosequencing

The fungi-specific primers ITS1F (CTTGGTCATTTAGAGGAA GTAA) (Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTC ATCGATGC) (White et al., 1990) were selected to target the ITS1 region. These primer pairs were modified for pyrosequencing by adding the forward Illumina Nextera adapter, a two-base-pair "linker" sequence, and a unique 7-bp barcode sequence to the 5′ end of the forward primer and the appropriate reverse Illumina Nextera adapter and linker sequence at the 5′ end of the reverse primer. PCR amplification was performed in a 25 μl reaction: 2.5 μl of 10 × reaction buffer, 10 μM of each primer, 2.5 mM dNTPs, 40 ng of template, and 0.625 units of Takara Pyrobest (Takara Biotechnology Co., Ltd., Japan). Amplifications were performed with the following temperature regime: 4 min of initial denaturation at 94°C, followed by 35 cycles of denaturation (94°C for 30 s), annealing (50°C for 45 s), extension (72°C for 1 min), and a final extension at 72°C for 7 min. The PCR products were purified using a PCR Purification Kit (Axygen Bio, USA). Then, paired-end sequencing was performed on an Illumina MiSeq sequencer at Personal Biotechnology Co., Ltd (Shanghai, China).

Quantification of the *Fusarium oxysporum* and Fungal Abundances

Real-time quantitative polymerase chain reaction (qPCR) was performed according to Chen et al. (2014) for quantifying the soil *Fusarium oxysporum* and fungi abundances using the SYBR Premix Ex Taq Kit on the ABI PRISM 7500 Real Time PCR System (Applied Biosystems, Germany). The 20 μl reaction mixture contained 10 μl of the *Premix Ex Taq*™ (2×) (Takara), 0.4 μl of each primer (10 μM), 0.4 μl of ROX Reference Dye II (50×), 2 μl of template DNA, and 6.8 μl of ddH_2O. The specific primer set of *F. oxysporum* and soil fungi was AFP308R (CGAATTAACGCGAGTCCCAAC)/ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Lievens et al., 2006) and ITS2 (GCTGCGTTCTTCATCGATGC)/ITS1F (CTTGGTCAT TTAGAGGAAGTAA), respectively. The thermal conditions were set as follows: 30 s at 95°C for initial denaturation, 40 cycles of 5 s at 95°C, and 34 s at 60°C. The standard curve was obtained using a 10-fold dilution series of plasmid DNA containing a fragment of the ITS region of *F. oxysporum* and ITS1 gene from the *F. oxysporum* f. sp. *vanillae* and soil samples, respectively. All amplifications were performed in triplicate. The specificity of the products was confirmed by a melting curve analysis and agarose gel electrophoresis. The copy numbers were $\log_{10}$-transformed to normalize the values prior to statistical analysis.

Pyrosequencing Data Analysis

After removing the adaptors and primer sequences, the raw sequences were assembled for each sample according to the unique barcode using QIIME (Caporaso et al., 2010). The split sequences for each sample were merged using FLASH V1.2.7 (Magoč and Salzberg, 2011), and low-quality sequences were then discarded using QIIME. The sequences retained for each sample were processed following the established UPARSE pipeline (Edgar, 2013). Briefly, the sequences with a quality score lower than 0.5 or a length shorter than 200 bp were removed. After discarding the singletons, the remaining reads were assigned to OTUs with a threshold of 97% identity level. Then, the chimera removal processes were performed. Finally, the fungal representative OTUs were classified using the UNITE database (Kõljalg et al., 2013).

The diversity within each individual sample was estimated using non-parametric Shannon diversity indices. Shannoneven was used to measure the evenness of each sample (Schloss et al., 2009). A principal coordinate analysis (PCoA) based on weighted UniFrac metric matrices was performed to explore the differences in fungal community structures among all of the soil samples (Lozupone et al., 2006). A permutational multivariate analysis of variance (Anderson, 2001) was performed to assess the effect of the cropping regime, soil compartment, and their interactions on the fungal community structure (abundance of OTUs and genus) using the adonis function of the R vegan package with 999 permutations.

Statistical Analyses

The soil physicochemical characteristics and vanilla *Fusarium* wilt DI between the black pepper-vanilla and vanilla monoculture systems were compared using Student's *t*-test. For other parameters in our study, one-way analyses of variance (ANOVA) with Turkey's HSD multiple range test were performed for multiple comparisons. All of the statistical analyses were performed using SPSS v20.0 (SPSS Inc., USA).

Sequence Accession Numbers

The sequence data have been deposited in the NCBI Sequence Read Archive (SRA) database with the accession number SRP062990.

RESULTS

Soil Physical and Chemical Properties in the Black Pepper-Vanilla and Vanilla Monoculture Systems

The results of soil physical and chemical properties are summarized in Table S1. When compared with the vanilla

monoculture system, black pepper-vanilla system presented a significantly ($P < 0.05$) higher available N content. In contrast, the vanilla monoculture system revealed higher soil pH and the contents of organic matter and available P.

Fusarium Wilt DI and Fungal Abundance in the Two Vanilla Cropping Systems

As shown in **Table 1**, the black pepper-vanilla system significantly reduced vanilla *Fusarium* wilt DI to 15.56%, whereas the value was over 60% in the vanilla monoculture system. The qPCR results showed that the ITS copies of *F. oxysporum* were significantly lower in the black pepper-vanilla system compared with those from the vanilla monoculture system in both bulk and rhizosphere soils (**Table 1**). Strikingly, the *F. oxysporum* populations significantly increased from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems. In addition, the fungal ITS gene copy numbers in bulk soil showed no significant difference between the black pepper-vanilla and vanilla monoculture systems (Table S2). Meanwhile, the fungal ITS gene copy numbers in the rhizosphere soil samples from the black pepper-vanilla system (8.15×10^9 copies g^{-1} soil) were significantly higher than those from the vanilla monoculture system (2.89×10^9 copies g^{-1} soil).

Overall Diversity of Fungal Community

After quality filtering, the pyrosequencing-based analysis of the fungal ITS1 genes resulted in the recovery of 1,260,032 high-quality sequences across the 12 samples (Table S3). The coverage from all samples was above 99%, indicating that the sequencing reads were sufficient for this analysis (Table S3). In both the bulk and rhizosphere soils, the black pepper-vanilla system had a significantly higher fungal diversity (Shannon) and evenness (Shannoneven) values than the vanilla monoculture system (**Figure 1**). In addition, in both the black pepper-vanilla and vanilla monoculture systems, the fungal community diversity and evenness significantly decreased from the bulk soil to the vanilla rhizosphere soil.

Fungal Community Composition

To verify the differences observed in the fungal communities from the black pepper-vanilla and vanilla monoculture systems, the relative abundances (RA) of the different classes and genera from the bulk and rhizosphere soils were compared (**Figures 2**, **3**). In the present study, fungal OTUs across the 12 soil samples were observed predominantly from the six classes (*Sordariomycetes*, *Eurotiomycetes*, unclassified *Zygomycota* class, *Dothideomycetes*, *Tremellomycetes*, and *Agaricomycetes*), accounting for 71.10% of the total fungal sequences. Compared with bulk soil, the relative abundance of the class *Sordariomycetes* in the vanilla rhizosphere soil significantly increased in both the black pepper-vanilla and vanilla monoculture systems. At the genus level, in the bulk soil, compared with the vanilla monoculture system, the black pepper-vanilla system had a higher relative abundance of *Mortierella*, *Aspergillus*, *Acremonium*, and *Chaetomium*. As for the rhizosphere soil, the

TABLE 1 | Vanilla *Fusarium* wilt disease incidence and pathogen abundance.

Cropping regime	Soil compartment	Disease incidence (%)	*Fusarium* RA	*F. oxysporum* RA	Log_{10} *F. oxysporum* ITS copies g^{-1} soil
Black pepper-vanilla system	Bulk soil (BB)	15.56 ± 3.85 b	6.79 ± 2.23 b	5.51 ± 2.75 b	4.80 ± 0.08 d
	Rhizosphere soil (BR)		10.36 ± 1.31 b	8.66 ± 1.15 b	5.60 ± 0.15 b
Vanilla monoculture system	Bulk soil (VB)	62.22 ± 10.08 a	10.23 ± 0.82 b	5.59 ± 0.37 b	5.13 ± 0.13 c
	Rhizosphere soil (VR)		26.18 ± 7.54 a	22.89 ± 6.80 a	6.18 ± 0.09 a

Values are means ± standard deviation (n = 3). RA, Relative abundance.
Means followed by the same letter for a given factor are not significantly different ($P < 0.05$; Turkey's HSD test where there are more than two treatment levels and Student's t-test where there are two treatment levels).

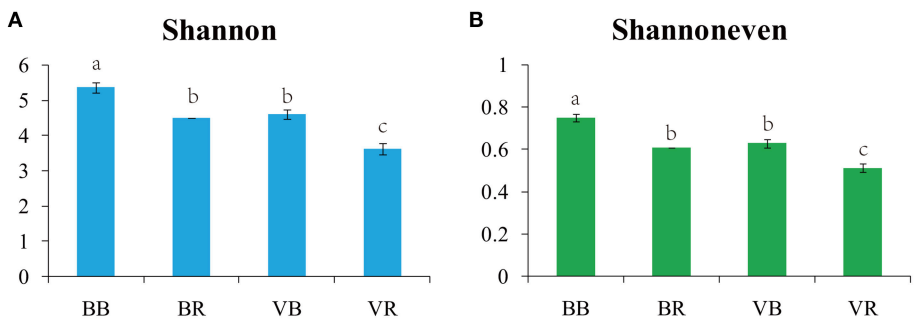

FIGURE 1 | Shannon diversity indices (A) and Shannoneven indices (B) for the bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems. Bars represent the standard deviation of the three replicates, and different letters above the bars indicate a significant difference at the 0.05 probability level according to the Turkey's HSD test. "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

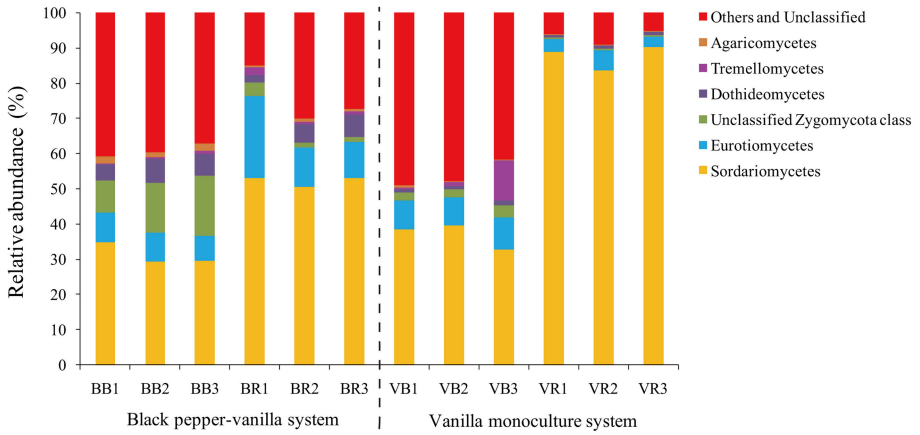

FIGURE 2 | Relative abundances of the main fungal classes in the bulk soil and the vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems. The "Others and Unclassified" comprised the unclassified and low-abundance classes (RA < 0.1%). "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

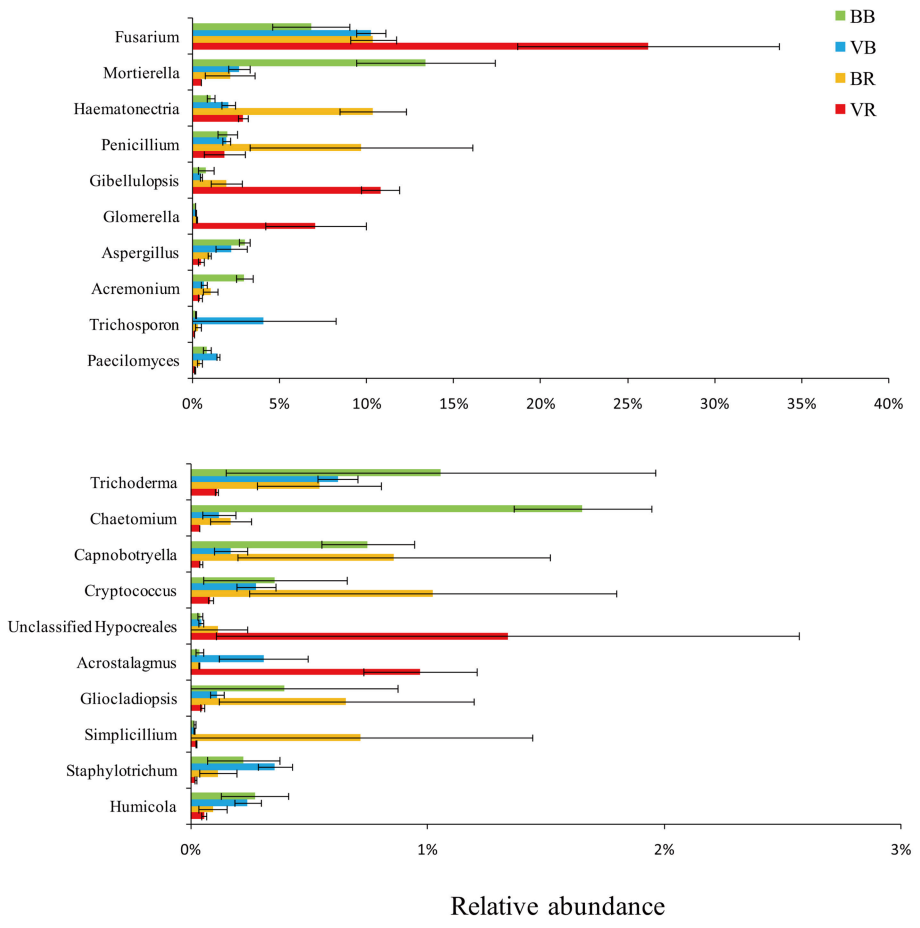

FIGURE 3 | Relative abundances of the top 20 fungal genera in bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems. Bars represent the standard deviation of the three replicates. "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

TABLE 2 | PERMANOVA analysis.

Source	Df	Abundance of Genera		Abundance of OTUs	
		Sums of sqs	Pseudo-F	Sums of sqs	Pseudo-F
Cropping regime (CR)	1	0.18	11.79***	0.42	7.64***
Soil compartment (SC)	1	0.25	15.80***	0.40	7.30***
CR × SC	1	0.09	5.54***	0.20	3.68***
Residuals	8	0.13	0.19	0.44	0.30

****Indicate significant correlations ($P < 0.001$).*

Fusarium genus was significantly more abundant in the vanilla monoculture system than in the black pepper-vanilla system; moreover, the relative abundance of *F. oxysporum* (OTU level) exhibited a similar trend (**Table 1**). In addition, the relative abundances of the genera *Haematonectria*, *Trichoderma*, and *Penicillium* were significantly higher in the black pepper-vanilla system with a lower *Gibellulopsis* abundance.

Fungal Community Structure

A permutational multivariate analysis of variance confirmed that the cropping regime, soil compartment, and their interactions were significant factors of variation for the fungal community structure in terms of both the relative abundance of OTUs and relative abundance of genera (**Table 2**).

To further compare the variations in fungal community structure between the black pepper-vanilla and vanilla monoculture systems samples, a UniFrac-weighted PCoA was employed. As shown in **Figure 4**, the bulk soil samples from the black pepper-vanilla system were clearly separated from the vanilla monoculture system, suggesting strong differences in fungal community structures between the different crop regime systems. In addition, the fungal communities in the rhizosphere soils from the two vanilla cropping systems were close together, suggesting that fungal community structures were seriously affected by the vanilla root system.

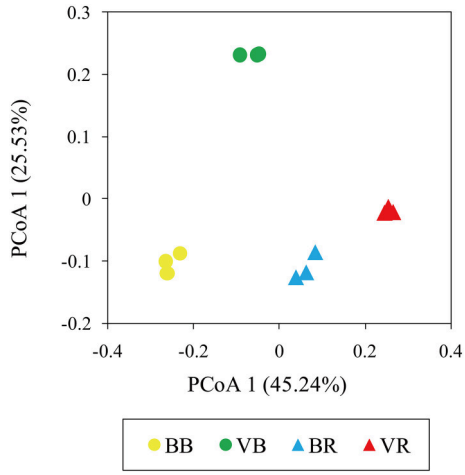

FIGURE 4 | UniFrac-weighted principle coordinate analysis of fungal community structures in the bulk soil and vanilla rhizosphere soil of the black pepper-vanilla and vanilla monoculture systems. "BB" and "VB" represent the bulk soil from the black pepper-vanilla system and the vanilla monoculture system, respectively. "BR" and "VR" represent the rhizosphere soil from the black pepper-vanilla system and the vanilla monoculture system, respectively.

DISCUSSION

Obstacles to the continuous cropping of vanilla have always been observed on Hainan Island (Xiong et al., 2015b). In the present study, pot experiments confirmed that long-term continuously cropped black pepper orchard soil showed significantly lower vanilla *Fusarium* wilt disease, implying that crop rotation is an effective management practice to reduce soil-borne plant disease in agro-systems (Wang et al., 2015). In addition, it will also help us to take advantage of the large area of black pepper continuous cropping soil in tropical China (Zu et al., 2014; Xiong et al., 2015a).

In this study, the black pepper-vanilla system had no effect on the fungal population abundance in the bulk soil. However, alpha diversity estimates of the fungal communities revealed that the black pepper-vanilla system had a significantly higher fungal diversity and evenness than the vanilla monoculture soil (**Figure 1**). The possible reasons are as follows: residues of black pepper decomposed in the soil and different root exudates could provide more available nutrient to soil microbes, thus improving species richness, heterogeneity, and diversity of the fungal community (Xuan et al., 2011). Furthermore, we could find a negative relationship between the soil fungal diversity and vanilla *Fusarium* wilt disease, which could support the idea that microbial diversity is a key factor in controlling pathogen invasion (van Elsas et al., 2012). In addition, in the present study, the fungal community diversity significantly decreased from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems; as explained by Mendes et al. (2014) plants can select a constant rhizosphere community from highly contrasting reservoirs of bulk soil communities.

The UniFrac-weighted PCoA analysis revealed significant variations in the bulk soil of fungal community structures between the black pepper-vanilla and vanilla monoculture systems (**Figure 4**). Our results agreed with the findings of Wang et al. (2015) where the crop regime system was the major determinant factor for microbial community structures. The significant variations in bulk soil community structures among the different cropping systems might be attributed to significant differentiations in soil physicochemical characteristics (Table S1), as soil physicochemical properties have significant impacts on microbial community structures (Lauber et al., 2008). Compared with the vanilla monoculture system, black pepper-vanilla system revealed a significantly higher available N content, as nitrogen play a pivotal role in plant growth and might indirectly enhance plant disease suppressiveness (Hayat et al., 2010). In addition, the UniFrac-weighted PCoA analysis suggested fungal community structures were also seriously affected by the vanilla root system, which was consistent with the many previous studies that plant

play a key role in shaping the microbial community structures in the rhizosphere (Philippot et al., 2013; Edwards et al., 2015).

The black pepper-vanilla system was shown to have a significant effect on the fungal community compositions in both bulk and rhizosphere soils. *Sordariomycetes* was the most abundant fungal class (**Figure 2**), which was generally consistent with the many early studies that found *Sordariomycetes* to be the most common fungal class in different agricultural systems (Chen et al., 2012; Li et al., 2014). Compared with bulk soil, the abundance of *Sordariomycetes* significantly increased in vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems, as reported by Zhang et al. (2006) who found that members of the *Sordariomycetes* are ubiquitous in virtually all ecosystems as pathogens and endophytes of plants.

Deeper taxonomic analyses were performed to explore the fungal community compositions of rhizosphere soil in the black pepper-vanilla system associated with vanilla growth. The black pepper-vanilla system showed significantly lower *Fusarium* and *F. oxysporum* abundance in the vanilla rhizosphere soil, which could be the most important reason for significantly lower vanilla *Fusarium* wilt disease in the black pepper-vanilla system (Pinaria et al., 2010). The *F. oxysporum* abundance is lower in the black pepper-vanilla system might be because that continuous cropping black pepper soil had not previously been used to cultivate vanilla, however, if vanilla is continuously cropped in that soil which could also increase the pathogen load eventually (Xiong et al., 2015b). In addition, in present study, the *F. oxysporum* populations significantly accumulated from the bulk soil to the vanilla rhizosphere soil in both the black pepper-vanilla and vanilla monoculture systems. Synthesized from the above results, we conclude that iterative crop rotation might be necessary to interrupt the accumulation of *F. oxysporum* abundance to suppress vanilla *Fusarium* wilt disease.

Some putatively plant-beneficial fungal groups, such as the genera *Trichoderma* and *Penicillium,* increased in the vanilla rhizosphere soil under the black pepper-vanilla system. *Trichoderma* spp. are known to have an effective antagonistic effect against vanilla *Fusarium* wilt disease (Jayasekhar et al., 2008; Vijayan et al., 2009). *Penicillium* is also a famous biocontrol agent for the biological control of *Fusarium* wilt disease (Larena et al., 2003); however, this has not yet been reported in vanilla systems. Moreover, *Haematonectria* was the most abundant genus, accounting for 10.33% of the total fungal genera in vanilla rhizosphere soil in the black pepper-vanilla system, which could occupy the rhizosphere niche to avoid pathogen invasion (Qiu et al., 2013). Combining the other variations in fungal genera in the black pepper-vanilla and vanilla monoculture systems and the complex interactions among these microorganisms could explain the status of vanilla *Fusarium* wilt disease in agro-ecosystems.

In conclusion, compared with the vanilla monoculture system, black pepper-vanilla system harbored a significantly lower abundance of *F. oxysporum* in vanilla rhizosphere soil, increased the putatively plant-beneficial fungal groups and the fungal diversity, which could explain the decrease in vanilla *Fusarium* wilt disease in the soil of the long-term continuously cropped black pepper orchard. These results suggested that sustainable agricultural management regime, such as crop rotation might be a meaningful strategy to prevent vanilla *Fusarium* wilt disease occurrence and will be our future research focus.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: W. Xiong, QZ, RL, HW, QS. Performed the experiments: W. Xiong, QZ, CX, W. Xun, JZ. Analyzed the data: W. Xiong, QZ, CX, JZ. Contributed reagents/materials/analysis tools: W. Xun, RL, HW, QS. Wrote the manuscript: W. Xiong, RL, HW, QS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00117

REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, L., Xun, W., Sun, L., Zhang, N., Shen, Q., and Zhang, R. (2014). Effect of different long-term fertilization regimes on the viral community in an agricultural soil of Southern China. *Eur. J. Soil Biol.* 62, 121–126. doi: 10.1016/j.ejsobi.2014.03.006

Chen, M., Li, X., Yang, Q., Chi, X., Pan, L., Chen, N., et al. (2012). Soil eukaryotic microorganism succession as affected by continuous cropping of peanut-pathogenic and beneficial fungi were selected. *PLoS ONE* 7:e40659. doi: 10.1371/journal.pone.0040659

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., et al. (2012). Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 21390–21395. doi: 10.1073/pnas.1215210110

Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for *basidiomycetes*-application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi: 10.1111/j.1365-294X.1993.tb00005.x

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60, 579–598. doi: 10.1007/s13213-010-0117-1

Hilton, S., Bennett, A. J., Keane, G., Bending, G. D., Chandler, D., Stobart, R., et al. (2013). Impact of shortened crop rotation of oilseed rape on soil and rhizosphere microbial diversity in relation to yield decline. *PLoS ONE* 8:e59859. doi: 10.1371/journal.pone.0059859

Jayasekhar, M., Manonmani, K., and Justin, C. G. L. (2008). Development of integrated biocontrol strategy for the management of stem rots disease (*Fusarium oxysporum* f. sp. *vanillae*) of *Vanilla*. *Agric. Sci. Dig. Abstr.* 28, 109–111. Available online at: http://www.indianjournals.com/ijor.aspx?target=ijor:asd&volume=28&issue=2&article=008

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi: 10.1111/mec.12481

Larena, I., Melgarejo, P., and De Cal, A. (2003). Drying of Conidia of *Penicillium oxalicum*, a Biological Control Agent against *Fusarium* Wilt of Tomato. *J. Phytopathol.* 151, 600–606. doi: 10.1046/j.0931-1785.2003.00772.x

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40, 2407–2415. doi: 10.1016/j.soilbio.2008.05.021

Li, X., Ding, C., Zhang, T., and Wang, X. (2014). Fungal pathogen accumulation at the expense of plant-beneficial fungi as a consequence of consecutive peanut monoculturing. *Soil Biol. Biochem.* 72, 11–18. doi: 10.1016/j.soilbio.2014.01.019

Lievens, B., Claes, L., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma, B. P. H. J. (2006). Detecting single nucleotide polymorphisms using DNA arrays for plant pathogen diagnosis. *FEMS Microbiol. Lett.* 255, 129–139. doi: 10.1111/j.1574-6968.2005.00074.x

Lozupone, C., Hamady, M., and Knight, R. (2006). UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 7:371. doi: 10.1186/1471-2105-7-371

Lu, L., Yin, S., Liu, X., Zhang, W., Gu, T., Shen, Q., et al. (2013). Fungal networks in yield-invigorating and -debilitating soils induced by prolonged potato monoculture. *Soil Biol. Biochem.* 65, 186–194. doi: 10.1016/j.soilbio.2013.05.025

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mendes, L. W., Kuramae, E. E., Navarrete, A. A., van Veen, J. A., and Tsai, S. M. (2014). Taxonomical and functional microbial community selection in soybean rhizosphere. *ISME J.* 8, 1577–1587. doi: 10.1038/ismej.2014.17

Metzker, M. L. (2010). Sequencing technologies—the next generation. *Nat. Rev. Genet.* 11, 31–46. doi: 10.1038/nrg2626

Minoo, D., Jayakumar, V. N., Veena, S. S., Vimala, J., Basha, A., Saji, K. V., et al. (2008). Genetic variations and interrelationships in *Vanilla planifolia* and few related species as expressed by RAPD polymorphism. *Genet. Resour. Crop Evol.* 55, 459–470. doi: 10.1007/s10722-007-9252-3

Navarro-Noya, Y. E., Gómez-Acata, S., Montoya-Ciriaco, N., Rojas-Valdez, A., Suárez-Arriaga, M. C., Valenzuela-Encinas, C., et al. (2013). Relative impacts of tillage, residue management and crop-rotation on soil bacterial communities in a semi-arid agroecosystem. *Soil Biol. Biochem.* 65, 86–95. doi: 10.1016/j.soilbio.2013.05.009

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Penton, C. R., Gupta, V. V., Tiedje, J. M., Neate, S. M., Ophel-Keller, K., Gillings, M., et al. (2014). Fungal community structure in disease suppressive soils assessed by 28S LSU gene sequencing. *PLoS ONE* 9:e93893. doi: 10.1371/journal.pone.0093893

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Pinaria, A. G., Liew, E. C. Y., and Burgess, L. W. (2010). *Fusarium* species associated with vanilla stem rot in Indonesia. *Australas. Plant Pathol.* 39, 176–183. doi: 10.1071/AP09079

Qiu, M., Li, S., Zhou, X., Cui, X., Vivanco, J. M., Zhang, N., et al. (2013). Decoupling of root–microbiome associations followed by antagonist inoculation improves rhizosphere soil suppressiveness. *Biol. Fertil. Soils* 50, 217–224. doi: 10.1007/s00374-013-0835-1

Sandheep, A. R., Aju, K., and Asok Ans Jisha, M. S. (2012). Biocontrol of *Fusarium* wilt of vanilla (*Vanilla planifolia*) using combined inoculation of *Trichoderma* sp. and *Pseudomonas* sp. *Int. J. Pharma Bio. Sci.* 3, 706–716. Available online at: http://www.ijpbs.net/vol-3/issue-3/bio/82.pdf

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Shokralla, S., Spall, J. L., Gibson, J. F., and Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Mol. Ecol.* 21, 1794–1805. doi: 10.1111/j.1365-294X.2012.05538.x

Tombe, M., and Sitepu, D. (1986). The control of stem rot of vanilla with fungicides. *Ed. Khusus Penelit. Tanam. Rempah Dan Obat* 11, 43–47.

van Elsas, J. D., Chiurazzi, M., Mallon, C. A., Elhottovâ, D., Krištùfek, V., and Salles, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1159–1164. doi: 10.1073/pnas.1109326109

Vijayan, A. K., Thomas, J., Dhanapal, K., and Thomas, J. (2009). Management of rot diseases of vanilla (*Vanilla planifolia* Andrews) using bioagents. *J. Plant. Crops* 37, 229–231. Available online at: http://www.cabdirect.org/abstracts/20113073569.html;jsessionid=F7D69B86DF6B31BA1BF18AEDAD901E98

Wang, B., Li, R., Ruan, Y., Ou, Y., Zhao, Y., and Shen, Q. (2015). Pineapple–banana rotation reduced the amount of *Fusarium oxysporum* more than maize–banana rotation mainly through modulating fungal communities. *Soil Biol. Biochem.* 86, 77–86. doi: 10.1016/j.soilbio.2015.02.021

Wang, B., Yuan, J., Zhang, J., Shen, Z., Zhang, M., Li, R., et al. (2013). Effects of novel bioorganic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of *Fusarium* wilt of banana. *Biol. Fertil. Soils* 49, 435–446. doi: 10.1007/s00374-012-0739-5

Wei, Z., Yang, X., Yin, S., Shen, Q., Ran, W., and Xu, Y. (2011). Efficacy of Bacillus-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. *Appl. Soil Ecol.* 48, 152–159. doi: 10.1016/j.apsoil.2011.03.013

White, T. J., Bruns, T., Lee, S., Taylor, J. W., and others (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc. Guide Methods Appl.* 18, 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1

Xiong, W., Li, Z., Liu, H., Xue, C., Zhang, R., Wu, H., et al. (2015a). The effect of long-term continuous cropping of black pepper on soil bacterial communities as determined by 454 pyrosequencing. *PLoS ONE* 10:e0136946. doi: 10.1371/journal.pone.0136946

Xiong, W., Zhao, Q., Zhao, J., Xun, W., Li, R., Zhang, R., et al. (2015b). Different continuous cropping spans significantly affect microbial community membership and structure in a vanilla-grown soil as revealed by deep pyrosequencing. *Microb. Ecol.* 70, 209–218. doi: 10.1007/s00248-014-0516-0

Xu, L., Ravnskov, S., Larsen, J., Nilsson, R. H., and Nicolaisen, M. (2012). Soil fungal community structure along a soil health gradient in pea fields examined using deep amplicon sequencing. *Soil Biol. Biochem.* 46, 26–32. doi: 10.1016/j.soilbio.2011.11.010

Xuan, D. T., Guong, V. T., Rosling, A., Alström, S., Chai, B., and Högberg, N. (2011). Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biol. Fertil. Soils* 48, 217–225. doi: 10.1007/s00374-011-0618-5

Zhang, N., Castlebury, L. A., Miller, A. N., Huhndorf, S. M., Schoch, C. L., Seifert, K. A., et al. (2006). An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* 98, 1076–1087. doi: 10.3852/mycologia.98.6.1076

Zu, C., Li, Z., Yang, J., Yu, H., Sun, Y., Tang, H., et al. (2014). Acid soil is associated with reduced yield, root growth and nutrient uptake in black pepper (*Piper nigrum* L.). *Agric. Sci.* 5, 466–473. doi: 10.4236/as.2014.55047

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Acid-Tolerant Moderately Thermophilic Methanotrophs of the Class *Gammaproteobacteria* Isolated From Tropical Topsoil with Methane Seeps

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Terrestrial tropical methane seep habitats are important ecosystems in the methane cycle. Methane oxidizing bacteria play a key role in these ecosystems as they reduce methane emissions to the atmosphere. Here, we describe the isolation and initial characterization of two novel moderately thermophilic and acid-tolerant obligate methanotrophs, assigned BFH1 and BFH2 recovered from a tropical methane seep topsoil habitat. The new isolates were strictly aerobic, non-motile, coccus-shaped and utilized methane and methanol as sole carbon and energy source. Isolates grew at pH range 4.2–7.5 (optimal 5.5–6.0) and at a temperature range of 30–60°C (optimal 51–55°C). 16S rRNA gene phylogeny placed them in a well-separated branch forming a cluster together with the genus *Methylocaldum* as the closest relatives (93.1–94.1% sequence similarity). The genes *pmoA, mxaF,* and *cbbL* were detected, but *mmoX* was absent. Strains BFH1 and BFH2 are, to our knowledge, the first isolated acid-tolerant moderately thermophilic methane oxidizers of the class *Gammaproteobacteria*. Each strain probably denotes a novel species and they most likely represent a novel genus within the family *Methylococcaceae* of type I methanotrophs. Furthermore, the isolates increase our knowledge of acid-tolerant aerobic methanotrophs and signify a previously unrecognized biological methane sink in tropical ecosystems.

Keywords: terrestrial, *Gammaproteobacteria*, methanotroph, acid-tolerant, moderately thermophilic, pMMO

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INTRODUCTION

Microorganisms in tropical ecosystems play a crucial role for biogeochemical cycling as well as controlling terrestrial greenhouse gas fluxes, and have therefore impact on global climate regulation. Methane is a strong greenhouse gas and the major biogenic source, which is the end product of microbial degradation of organic matter in anoxic environments. Major sources of abiogenic methane are underground reservoirs in geothermal regions, where methane is released to the atmosphere through seeps, gas venting, and degassing of spring water (Etiope and Klusman, 2002; Aronson et al., 2013; Nazaries et al., 2013). In some regions, like the gas fields in Northeast Bangladesh, release of natural methane from tropical soils are caused by previous surface gas

blowouts (Khan and Nasir, 2014). Such methane seeps influence the structure and function of microbial communities, and have importance for the global carbon cycle. Methane-oxidizing bacteria (MOB) or methanotrophs serve as a methane sink that suppress methane emissions to the atmosphere from various ecosystems, and contribute extensively to the global methane budget. Methanotrophic community structure and activity have been studied in terrestrial tropical habitats such as rice paddy fields (Alam and Jia, 2012; Dianou et al., 2012), peat soils (Arai et al., 2014), and upland soils (Knief et al., 2005) as well as in tropical shallow methane seep sediments (Wasmund et al., 2009). These studies, which have applied analyses of methane fluxes and populations of methanotrophs, support evidences for diverse communities of aerobic methanotrophs and the existence of novel uncultured MOBs. Although, several methanotrophs from tropical ecosystems have been isolated (Geymonat et al., 2011; Islam et al., 2015; Khalifa et al., 2015), but knowledge about methanotrophs and their responses to environmental factors in such ecosystems is still limited (Aronson et al., 2013). Furthermore, no previous studies have reported on methanotrophic communities in high temperature tropical soils (around 50°C) with high methane concentrations due to surface gas blowouts. Such investigations are essential to understand the microbial impact and the effect of environmental factors on methane fluxes. In particular, studies of isolated methanotrophs from such tropical ecosystems will expand our knowledge about the genetics, biochemistry, and ecophysiology of this functional group.

The methane oxidizing microbes constitute a unique group defined by their ability to utilize methane as sole source for carbon and energy, and they are isolated from a wide variety of habitats (Hanson and Hanson, 1996). Several clusters of uncultivated methane oxidizers have been detected in both tropical and temperate ecosystems, indicating the need for further cultivation efforts to obtain new pure cultures of methanotrophs (Knief, 2015). Until now, twenty two genera of proteobacterial methanotrophs (Deutzmann et al., 2014; Hoefman et al., 2014; Khalifa et al., 2015; Tavormina et al., 2015) and two genera of verrucomicrobial extreme acidophilic methanotrophs (Op den Camp et al., 2009; Sharp et al., 2014b; van Teeseling et al., 2014) are validly described. In the phylum *Proteobacteria*, MOB are assigned to four families *Methylothermaceae*, *Methylococcaceae*, *Methylocystaceae* and *Beijerinckiaceae*, which are described based on phylogeny, chemotaxonomy, arrangement of intracytoplasmic membrane (ICM), DNA mol% G+C, and pathways for carbon assimilation (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008; Hirayama et al., 2014).

Commonly used molecular markers for MOB are the key functional marker genes *pmoA* (the particulate methane monooxygenase), *mmoX* (the soluble methane monooxygenase), and *mxaF* (the methanol dehydrogenase). In particular, the *pmoA* gene, encoding the 27 kDa polypeptide of the particulate methane monooxygenase, has been used as a most frequently phylogenetic marker for identifying aerobic methanotrophs except those in the family *Beijerinckiaceae*. The *mxaF* gene was also suggested as a functional and phylogenetic marker for proteobacterial methanotrophs and methylotrophs in natural environments (McDonald and Murrell, 1997; McDonald et al., 2008; Lau et al., 2013). Another functional gene, *cbbL*, encodes the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is a key enzyme responsible for autotrophic CO_2 fixation of the Calvin-Benson-Bossham (CBB) cycle. The *cbbL* gene has been frequently applied for analyzing marine and hypersaline microbial communities (Tourova et al., 2010) as well as for identification of methanotrophic *Methylococcus*-like strains and *Methylocaldum* species (Baxter et al., 2002).

Methylococcus capsulatus was the first described heat-tolerant methanotroph within the family *Methylococcaceae*, growing at temperatures up to 50°C and at pH range 5.5–7.5 (Foster and Davis, 1966; Whittenbury et al., 1970). Several moderately thermophilic methanotrophs of the class *Gammaproteobacteria*, such as *Methylocaldum szegediense*, *Methylocaldum tepidum*, *Methylocaldum gracile*, *Methylocaldum* sp. strain H-11, *Methylocaldum* sp. strain O-12, *Methylocaldum marinum* and *Methylothermus thermalis* (Bodrossy et al., 1997; Eshinimaev et al., 2004; Tsubota et al., 2005; Takeuchi et al., 2013) were subsequently isolated from thermal environments and marine sediments. In addition, a true thermophilic methanotroph named strain HB, which grew on methane up to 72°C, was isolated from underground hot springs in Hungary (Bodrossy et al., 1999). Sequence comparison indicated that the *pmoA* gene was most closely related to known *Methylococcus* and *Methylocaldum* species. Unfortunately, this strain is no longer available (Tsubota et al., 2005). Moreover, using stable-isotope probing in combination with 16S rRNA gene pyrotag sequencing, *Methylocaldum* species were identified in sediments of warm geothermal springs ranging in temperature from 22 to 45°C (Sharp et al., 2014a). Recently, three mesophilic *Methylocaldum*-like methanotrophs (growth range 8–35°C) of the family *Methylococcaceae* were isolated from different geographic regions, and these isolates fell into a cluster consisting of the genera *Methylocaldum-Methylococcus-Methyloparacoccus-Methylogaea* (Islam et al., 2015).

Extreme acidophilic methanotrophs of the phylum *Verrucomicrobia* have been discovered by cultivation-dependent and -independent approaches (Sharp et al., 2014b; van Teeseling et al., 2014). Three thermoacidophilic verrucomicrobial methanotrophs (pH growth range 0.5–6.0 and optimum temperature 55–60°C) have been described, thus extending the phylogeny of true thermoacidophilic methanotrophs beyond the phylum *Proteobacteria*. The verrucomicrobial methanotrophs belong to the family *Methylacidiphilaceae*, and were obtained from extreme geothermal environments at different geographic regions. (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008; Op den Camp et al., 2009). Mesophilic verrucomicrobial methane oxidizers have also been characterized, which shows that methanotrophs in this phylum are more diverse and widespread than earlier anticipated (van Teeseling et al., 2014). Slightly acidophilic methanotrophs belonging to the genera *Methylocella*, *Methylocapsa,* and *Methyloferula* of the class *Alphaproteobacteria* are abundant in peat bog ecosystems and able to grow between pH 4.2 and 7.5 (Dedysh et al., 2007; Dedysh, 2009; Vorobev et al.,

2011). In addition, two strains of acid-tolerant or acidophilic type I methanotrophs, *Methylomonas* strain M5 and strain M200 of the family *Methylococcaceae* were isolated from peat ecosystems, and they are able to grow below pH 4.5 (Kip et al., 2011). Recently, an acid-tolerant type I methanotroph, *Methylomonas paludis* strain MG30, was also isolated from an acidic *Sphagnum* peat bog in north-eastern Russia and had a pH growth range of 3.8–7.3 (Danilova et al., 2013).

Hitherto, no true thermoacidophilic or acid-tolerant moderately thermophilic gammaproteobacterial methanotrophs have been described. The present study describes the recovery and initial characterization of two novel methane oxidizers belonging to the class *Gammaproteobacteria* isolated from a methane-rich tropical topsoil.

MATERIALS AND METHODS

Sampling

Sampling was performed in June 2007, and again in November 2009. Tropical topsoil (5 cm depth) was collected from the side of a small cave, which was heated by a flame of natural methane gas leakage or surface gas blowout. The place is called Horipur (village: Utlarpar) and is situated about 20 km from Sylhet in northeast Bangladesh (24° 98′07″ N, 92° 03′29″ E; **Figure 1**). The *in situ* soil temperature was measured by a digital temperature sensor (Digitron, 2000T, Sifam Instruments, UK). The pH of topsoil (1:1, soil:water) was measured by a pH meter (MP220, Mettler Toledo). Soils were placed in Falcon tubes and frozen at −20°C on the sampling day.

Isolation and Cultivation

To enrich for moderately thermophilic MOB, low-salt mineral medium supplemented with NH_4Cl (0.1 g L^{-1}; LMA) was used. This medium is 10 times more diluted than the AMS medium (Whittenbury et al., 1970). No vitamins were included in the medium. The pH was adjusted to 6.0 and 6.5 with 1 M HCl or 1 M NaOH. In addition, low-salt mineral medium with NH_4Cl replaced by KNO_3 (0.1 g L^{-1}) was applied for methanotrophic enrichments. Three gram of soil was added to 20 mL medium in 120 mL sterile serum bottles. The bottles were closed with a butyl rubber cap with an aluminum crimp seal. A mixture of methane (purity 99.5%, Yara Praxair, Oslo, Norway) and air was added aseptically through a syringe to achieve 80 and 20% concentration of methane and air, respectively, in the headspace. The flasks were shaken at 125 rpm on a rotary shaker kept at 50°C. After 1 week incubation, the medium became visibly turbid and growth of the enrichment cultures was verified by phase-contrast microscopy (Eclipse E400 microscope, Nikon Corporation, Tokyo, Japan). Then, 1 mL of the cultures were transferred to fresh LMA and incubated at the same conditions. After five passages of the enrichments, the cultures were serial diluted (10^{-5} to 10^{-8}), and 0.1 mL aliquots of each dilution were spread onto plates containing a mixture of LMA and gelrite (20 g L^{-1}; Gelzan^{TM} CM, Sigma-Aldrich, Corporation, St. Louis, MO, USA) or agar (Difco). The plates were incubated at 50°C in jars filled with a methane/air (4:1) gas mixture. Individual colonies were picked and re-streaked onto fresh plates and re-incubated. Finally, single colonies were transferred to fresh liquid LMA and incubated for 1 week with methane and air.

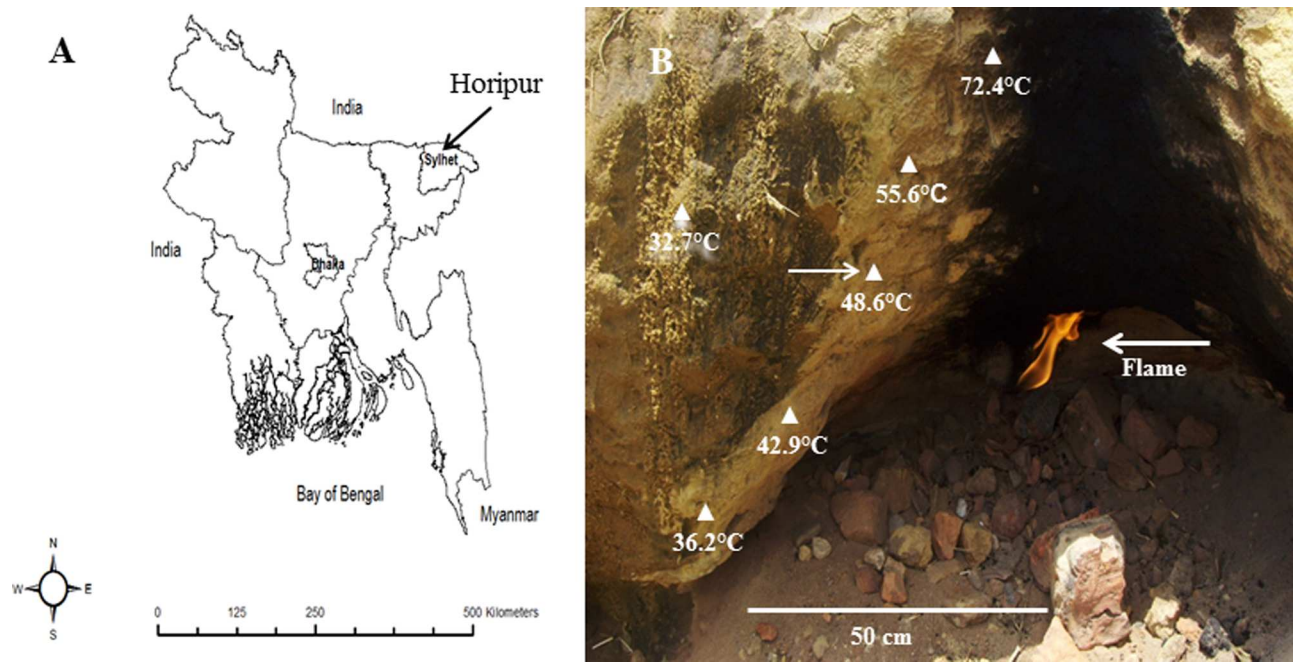

FIGURE 1 | Sampling site. (A) Map of Bangladesh showing the sampling place (ArcGIS Software; www.esri.com/software/arcgis). **(B)** A close-up photograph (with temperature gradient) of terrestrial tropical topsoil by leakage natural methane gas at Horipur, Sylhet, north-east Bangladesh. The arrow indicates the sampling site.

Purity Verification of Isolates and Electron Microscopy

The purity of isolates was examined by phase-contrast microscopy, and further verified by formation of uniform colonies on LMA gelrite plates and absence of growth of heterotrophs in LMA supplemented with Luria-Bertani broth (1–5% v/v), glucose (10 mM), acetate (18 mM), pyruvate (10 mM), succinate (10 mM), ethanol (17 mM), yeast extract (0.1%), and by streaking onto R2A (Reasoner's 2A) agar plates (van der Linde et al., 1999). The isolation process was monitored by polymerase chain reaction (PCR) amplification and sequencing of the partial 16S rRNA gene until each of the isolates gave a clean and reproducible sequence read. Exponentially growing cultures of strain BFH1 were collected by centrifugation and applied for transmission electron microscopy analysis (a Jeol-1230 electron microscope, at 60 KV, Tokyo, Japan), as described previously (Islam et al., 2015).

Utilization of Carbon and Nitrogen Sources

Capability to utilize various organic compounds was tested in liquid LMA medium supplemented with the following autoclaved or filter-sterilized substrates at 10 mM concentration: urea, acetate, glucose, pyruvate, lactate, malate, ethanol, succinate, sucrose, fructose, maltose, mannitol, and sorbitol. Growth on the C1 substrates methanol, formate, methylamine, and formaldehyde were tested at concentrations from 0.03 to 0.2% (v/v). Luria-Bertani broth and yeast extract were also tested as supplements to LMA. During incubation, bottles were capped with butyl-rubber stoppers to prevent vaporization. The growth of the strains was also tested with nitrogen-free LMA (without NH_4Cl or KNO_3) in triplicate 120-mL serum bottles where N_2 from the air (20% air in the headspace) was the only N-source. The bottles were incubated for 2 weeks.

Optimum pH, Temperatures, and Salt Concentrations

The temperature range and the optimum temperature for growth were tested at 20, 25, 28, 30, 35, 37, 40, 45, 48, 50, 54, 58, 60, 62, 65, and 70°C (growth at pH 6.0). In order to evaluate the effects of pH we measured the growth rates of the isolates at the optimum temperature 51°C by counting cells in the phase-contrast microscope. Growth rates were assessed at the following pH values: 3.0, 3.5, 4.0, 4.2, 4.3, 4.5, 5.0, 5.5, 6.0, 6.5, 6.8, 7.2, 7.5, 7.8, 8.0, 8.5, 9.0. To determine the salt-dependence of growth, NaCl (0.1, 0.5, 1.0, 1.5, 2.0, and 3.0%, w/v) was added to the medium. The generation time was calculated during exponential growth phase. The optical cell density (at 600 nm) was measured at optimum temperature (51°C) and at pH 6.0. Antibiotic sensitivity was tested and the following antibiotics ($\mu g\ mL^{-1}$) were used: ampicillin, 10; tetracycline, 10; kanamycin, 30; streptomycin, 10; erythromycin, 10; and nalidixic acid, 30. Growth was evaluated after 10 days incubation.

Acetylene Inhibition Test, Naphthalene Assay, Fatty Acid Profiles, and DNA mol% G+C

The naphthalene-oxidation assay, testing for the presence of soluble form of methane monooxygenase (sMMO), was performed with a liquid LMA culture without copper (Graham et al., 1992). *M. capsulatus* strain Bath was used as positive control of the assay. The effects of acetylene were examined by adding 4% (vol/vol) acetylene in the headspace of three replicate flasks containing cultures of the isolates in early exponential phase growing in LMA. To verify the acetylene inhibition test, *M. capsulatus* strain Bath and *Methylacidiphilum kamchatkense* strain Kam1 were used as positive controls (Islam et al., 2008). Phospholipid fatty acid (PFLA) and DNA mol% G+C analyses were performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

PCR Amplification and Southern Blot Hybridization

Genomic DNA was extracted with GenElute bacterial genomic DNA kit (Sigma-Aldrich) and used as template for PCR amplification of the 16S rRNA gene using the universal bacterial primers 27f and 1492r (Weisburg et al., 1991). The functional genes *pmoA*, *mmoX*, *mxaF*, and *cbbL* were amplified using primers listed in Supplementary Table S1. PCR amplifications were carried out in 50 μL volumes in a Veriti 96 well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) using Dynzyme^{TM} High-Fidelity DNA Polymerase (Finnzymes, Finland). All PCR reactions were performed as described previously (Islam et al., 2015). Genomic DNA was also used for Southern blot analysis and DNA from *M. capsulatus* strain Bath, and *M. kamchatkense* strain Kam1 were applied as positive and negative controls, respectively. Genomic DNA was digested with EcoRI and HindIII and separated by agarose gel electrophoresis and blotted onto Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). Hybridization probes were prepared by PCR using the *pmoA* and *mmoX* primer sets (Supplementary Table S1) and labeled with [α-^{32}P]dCTP using DNA label kit (Amersham Biosciences) as previously described (Baxter et al., 2002; Islam et al., 2008).

Phylogenetic Analysis

The nucleotide sequences of the functional genes were translated into amino acid sequences using the ExPASy Translate tool[1]. 16S rRNA sequences and the deduced protein sequences (PmoA, MxaF, and CbbL) were compared with available sequences in the GenBank database using BLASTn and BLASTp (the NCBI tools), respectively. Phylogenetic analyses based on 16S rRNA and PmoA sequences were carried out by aligning sequences using CLUSTAL W algorithm as implemented in MEGA6 software package (Tamura et al., 2013). Phylogenetic trees were constructed by different methods such as the Neighbor-Joining, Maximum-Likelihood, and Minimum-Evolution. Distances were

[1]http://web.expasy.org/translate

determined using either Kimura 2-parameter models, Jones-Taylor/Thornton or Maximum composite likelihood method also implemented in MEGA6 software. The 16S rRNA, particulate methane monooxygenase (*pmoA*), methanol dehydrogenase (*mxaF*), and the ribulose-1,5-bisphosphate caboxylase/oxygenase (*cbbL*) gene sequences of strains BFH1 (GQ130271, GQ130270, GQ130269, KP878519) and BFH2 (KP828774, KT921321, KT921322, and KT932010) respectively, were deposited in the GenBank.

RESULTS

Isolation of Acid-Tolerant Moderately Thermophilic MOB

The *in situ* temperature and pH at the sampling site were 48.6°C and 5.0, respectively. Very poor growth of enrichment cultures of methanotrophic bacteria was observed in a low-salt mineral medium supplemented with KNO_3 whereas better growth was observed in LMA medium (with NH_4Cl). The enrichment cultures in LMA medium reached a density of about 10^8 cells mL^{-1} after a second transfer. Strains BFH1 and BFH2 were isolated after subculturing in fresh LMA five times and growth of single colonies on gelrite plates. Small white colonies of the strains BFH1 (about 0.8–1 mm in diameter) and BFH2 (about 1–1.4 mm in diameter) were observed on gelrite plates after 10 days incubation in methane:air (80:20) atmosphere. On agar plates, no colonies appeared even after 3 weeks of incubation. Growth with methane or methanol (0.05%) in liquid cultures first developed white turbidity and later became semitransparent. No growth of the strains was observed in the absence of methane or in the presence of methane under anaerobic conditions.

Morphological Properties

Morphological characteristics of strains BFH1 and BFH2 as compared to thermotolerant, thermophilic, and acid-tolerant proteobacterial methanotrophs are presented in **Table 1**. Cells of the isolates (in exponential and early stationary phase) were non-motile and had slightly elongated coccus-shape, 1.0–2.0 μm in length and 1.0–1.3 μm in diameter. They usually occurred single or in pairs, and did not form chains or aggregates (**Figure 2A**, strain BFH1). No cyst-like cells were detected. Cells were Gram-negative and reproduced by binary fission. By transmission electron microscopy of BFH1, extensive ICM structures, a typical feature of pMMO-possessing proteobacterial methanotrophs, was confirmed (**Figures 2B,C**). No flagella were observed.

Physiological Characteristics and Fatty Acid Profile

Physiological characteristics of strains BFH1 and BFH2 are presented in **Table 1**. The isolates were unable to grow on substrates containing multi-carbon compounds or in complex media, and utilized only methane and methanol as carbon and energy source. They could utilize methanol at a wide range

TABLE 1 | Comparison of the major characteristics of the acid-tolerant moderately thermophilic strains BFH1 and BFH2 with themotolerant, thermophilic, and acid-tolerant methane oxidizing bacteria of the families *Methylococcaceae* and *Methylothermaceae*.

Characteristic	Strains BFH1 and BFH2	[1]*Methylocaldum* spp.	[2]*Methylothermus thermalis*	[3]*Methylococcus capsulatus*	[4]*Methylomagnum ishizawai*	[5]Acid-tolerant strain M200
Cell morphology	Coccoids	Rods-pleomorphic	Coccoids	Coccoids	Rods	Cocci
Acidophilic condition	Acid-tolerant	Neutrophilic	Neutrophilic	Neutrophilic	Neutrophilic	Acid-tolerant
ICM arrangement	Type I	Type I	Type I	Type I	Type I	Type I
Motility	–	–	–	–	–	+
Cyst formation	–	+	–	–	+	–
Pigmentation	White	Brown/cream	White	Yellow	White	Pink
Methane oxidation	pMMO	pMMO	pMMO	pMMO/sMMO	pMMO	pMMO/PxmA
mmoX	–	+[a]	–	+	+	–
*mxa*F	+	+	+	+	nr	nr
cbbL	+	+	+	+	nr	nr
N_2-fixation	+	+	–	+	–	+
Temperature (optimal) °C	30-60 (51-55)	20-61 (42-55)	37-67 (57-59)	20-47 (42-45)	20-37 (31-33)	4-30
Growth of pH (optimal)	**4.2-7.5 (5.5-6.0)[b]**	**6.0-8.5 (7.0-7.2)**	**6.5-7.5 (6.8)[c]**	**5.5-7.5 (6.5)**	**5.5-9.0 (6.8-7.4)**	**4.1-7.0 (5.5)**
Growth on methanol (0.1%)	+	–	+	+	–	+
Vitamin required	–	–	–	–	–	–
G+C content (mol%)	62.7	58.5	62.5	62.5	64.1	52.2[d]
Isolation source (pH)	Tropical topsoil (pH 5.0)	Manure, silage (pH 6.0)	Hot spring (pH 6.0)	Hot spring (pH 6.0)	Rhizosphere soil (pH 6.8)	*Sphagnum* mosses (pH 3.8–4.3)

Reference for strains: [1]Bodrossy et al., 1997 and Eshinimaev et al., 2004; [2]Tsubota et al., 2005; [3]Bowman et al., 1993; [4]Khalifa et al., 2015; [5]Kip et al., 2011. +, positive results; –, negative results; nr, not reported; ICM, intracytoplasmic membrane. [a]Only Methylocaldum marinum strain S8^T (Takeuchi et al., 2013) possessed mmoX (encodes soluble methane monooxygenase). [b]Growth on LMA with methane or methanol of strain BFH2 was observed at pH range 4.5-7.5. [c]Methylothermus subterraneus strain HTM55^T grew at pH 5.2–7.5 (Hirayama et al., 2011). [d]16S rRNA, pmoA, and pxmA genes sequences were used for computing measurement of DNA G+C content (mol%).

Significance of bold values: to compare pH growth among methane oxidizers.

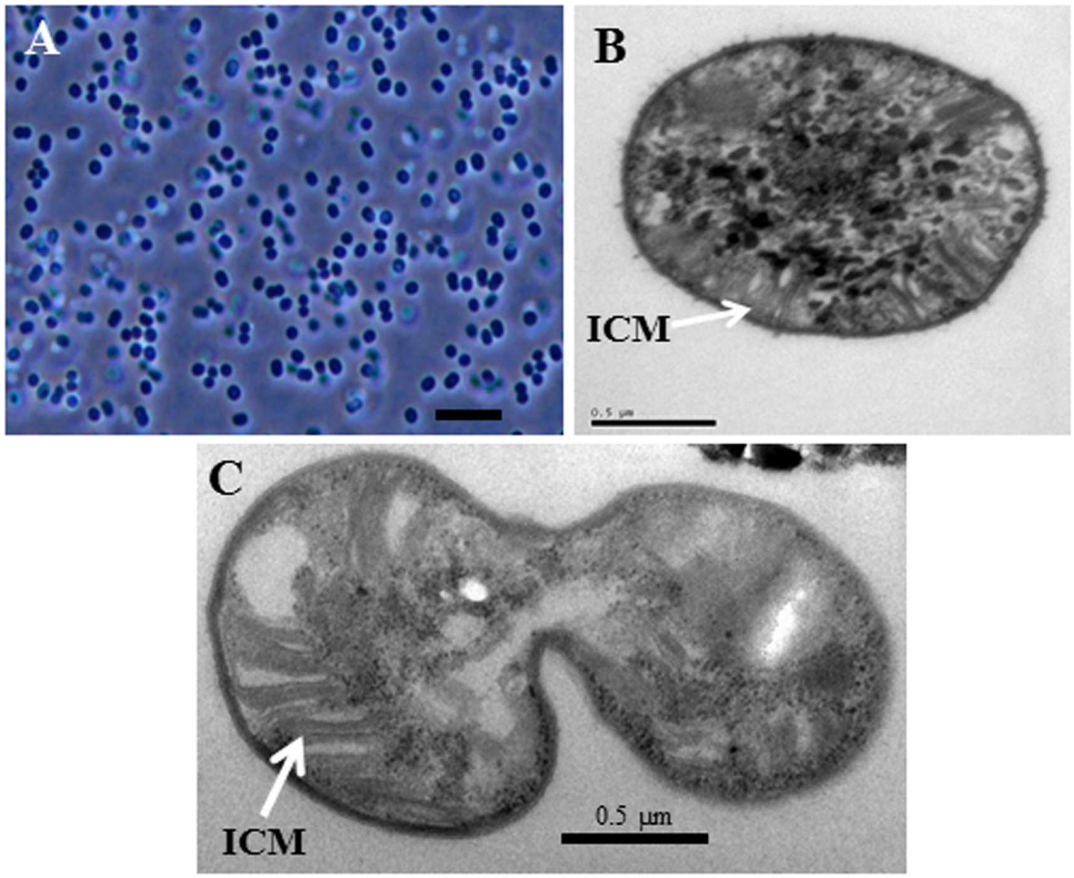

FIGURE 2 | Morphology of strain BFH1. (A), Phase-contrast photomicrograph of cells grown in LMA medium under methane at 51°C for 5 days. **(B,C)** Transmission electron micrograph of the strain BFH1. Ultrathin sections showing intracytoplasmic membrane arrangements (ICM). Bars, 3 μm **(A)**, 0.5 μm **(B,C)**.

of concentrations (0.05-0.2%), with an optimum concentration between 0.1 and 0.15%. Vitamins were not required for growth. Cells used ammonium or nitrate as nitrogen sources. Better growth was observed with NH_4Cl than with KNO_3 or $NaNO_3$ in LMA medium. Growth in nitrogen-free liquid medium was observed but it was not as good as with NH_4Cl. The strains were able to grow at NaCl concentrations up to 0.5% (w/v). The growth temperature range of both strains was 30-60°C (no growth occurred at 25 or 65°C) and the optimum growth temperature was 51–55°C. Growth of strains BFH1 and BFH2 was observed at pH range 4.2–7.5 and 4.5–7.5, respectively, and no growth was observed at pH 4.0 or 7.8. Fastest growth was observed at pH between 5.5 and 6.0 for BFH1, and 5.7 and 6.2 for BFH2 (Supplementary Figure S1). The estimated generation times of strains BFH1 and BFH2 under optimum conditions were 21 h (specific growth rate, 0.033 h^{-1}) and 19 h (specific growth rate, 0.036 h^{-1}), respectively. All the tested antibiotics inhibited growth and the naphthalene oxidation assay was negative. Acetylene is often applied as inhibitor for the methane oxidation process and growth on methane was completely blocked after addition of 4% (vol/vol) acetylene to the headspace. The fatty acid compositions of strain BFH1 and other related thermophilic, thermotolerant, and acid-tolerant methanotrophs are presented in **Table 2**. The major fatty acids in the strain BFH1 were C16:0 (54.38%), C17:0*cyc* (26.33%) and C16:1ω7 (13.83%).

Analysis of Functional Genes and Phylogenetic Characterization

Polymerase chain reaction products were obtained from the functional genes *pmoA*, *mxaF*, and *cbbL* for both BFH1 and BFH2 (**Table 1**), whereas PCR amplification of the *mmoX* gene gave negative results. The absence of *mmoX* was verified by Southern hybridization using *mmoX* probe, which gave no signal with genomic DNA of the two strains (Supplementary Table S2). Phylogenetic analysis of the nearly full-length (1409 bp of strain BFH1 and 1429 bp of strain BFH2) 16S rRNA sequences revealed that both strains belonged to the class *Gammaproteobacteria*, and showed 97.8% sequence identity to each other. The strains clustered with moderately thermophilic and thermotolerant methanotrophs in the genera *Methylocaldum*, *Methylococcus*, and the mesophilic genera *Methyloparacoccus* and *Methylogaea* belonging to the family *Methylococcaceae* (**Figure 3**). BLASTn search of 16S rRNA genes revealed that the strains showed 97–99% sequence identity

TABLE 2 | Cellular PLFA profiles comparison of the strain BFH1 and other phylogenetically related methanotrophic genera or species.

Fatty acids	1	2	3	4	5
C13:1					0.27
iC14:0	0.59		1.24		
C14:0	0.78	1.97		0.8–6.2	15.8
C14:0 2-OH			0.33		
C15:	0.80	3.51	2.07	0–1.7	1.56
C15:1ω8					0.22
C15:1ω6	0.12		0.17		
iC16:0	0.26				
C16:1ω11					5.46
C16:1ω7c	**13.83**	**3.46**	**10.6–23.1**	**47.3**	
C16:1ω6c				3.9–12.3	8.03
C16:1ω5c	0.37			3.2–9.0	
C16:1ω5t				1.8–6.0	
C16:0	**54.38**	**63.67**	**37.24**	**33.5–56.0**	**19.6**
C16:1		11.90			
C16:0 3-OH	0.28	0.64			1.78
9-*o*-Me-C16:0		4.62			
C17:0*cyc*	**26.33**	**8.99**	**4.71**	**0–14.0**	
aC17:0		0.68			
C17:0	0.46	0.34	2.52		
C17:1		0.43	0.19		
C17:1ω6	0.62	0.26			
C17:1ω7c				0–1.9	
9-o-Me-C17:0		0.60			
11-0-Me-17:0		0.60			
C17:0	0.31				
iC18:0		0.26		0.6–1.8	
C18:0		0.17	1.74	0–2.1	
C18:1ω7c	0.71		0.35	0–6.5	
C18:1ω9c	0.62		35.16	0–2.9	
C19:0*cyc*				0.6–1.8	
C19:1*cyc*		1.37	2.41		

Strains: (1) Strain BFH1 (data from this study); (2), Methylocaldum sp. O-12 (Eshinimaev et al., 2004); (3) Methylothermus thermalis MYHT^T (Tsubota et al., 2005); (4) Methylococcus capsulatus (data from Bowman et al., 1993); (5) Methylomagnum ishizawai RD11D-Pr^T (Khalifa et al., 2015). Values are given as a percentage of total fatty acids.

Significance of bold values: to compare major fatty acids among methane oxidizers.

to clones of uncultured bacteria from industrial sugarcane bagasse feedstock piles (GenBank Accession No. HM362597, HM262490, HM362577, HM362564, HM362458, HM362551, HM362534–36, HM352524, HM352510; Rattanachomsri et al., 2011). The closest isolated relatives were the thermophilic methanotrophs *Methylocaldum* sp. dr65 (identity 97.6–99.7%) and *Methylocaldum* sp. r6f (identity 97.6–99.7%), isolated from landfill cover soils, *Methylocaldum* sp. E10a (identity 94.9–95.3%; Knief and Dunfield, 2005), and *Methylocaldum* sp. 05J-I-7 (identity 95.6%; GenBank Accession No. EU275146) isolated from landfill upland soil. The strains BFH1 and BFH2, however, formed a separate branch from *Methylocaldum* spp., which was supported by a high bootstrap value. Furthermore, pairwise sequence analysis of 16S rRNA genes of BFH1 and BFH2 with the closest described relatives showed only 93.1–94.1% sequence identity to *Methylocaldum* species (*M. szegediense* OR2^T, *M. gracile* VKM 14L^T, *M. tepidum* LK6^T, *Methylocaldum marinum* S8^T), 92.0–92.7% to *Methyloparacoccus* spp., 90.2-92.4% to *M. capsulatus* strain Bath, 90–90.8% to *Methylogaea oryzae* E10^T, 87.9–88.4% to *M. thermalis* MYTH^T and 88.0–88.2% to acid-tolerant type I methanotrophic strain M200 (Supplementary Table S3). This indicates that our strains most probably represent a new genus of type I gammaproteobacterial methanotrophs. All 16S rRNA phylogenetic trees, Neighbor-Joining tree (**Figure 3**), Maximum-Likelihood tree (Supplementary Figure S2), and Minimum-Evolution tree (Supplementary Figure S4), exhibited the same topologies for strain BFH1 and BFH2, indicating that they were not members of other described methanotrophic genera in the families *Methylothermaceae* or *Methylococcaceae*.

BLASTp search of the PmoA amino acid sequence of the strain BFH1, showed 94% identity to uncultured methanotrophic clones from planted rice-field soil (GenBank Accession No. CA098534, AGO14814, and AFL65442), activated sludge (GenBank Accession No. BAG12175), and rice wetland (CBI68832). The closest cultured relatives of BFH1 and BFH2, based on PmoA sequences, were *Methylocaldum* spp. (similarity 94.9–96.8%), *Methyloparacoccus* spp. (94.9%), *M. capsulatus* strain Bath (93.7%), *M. oryzae* E10^T (93.1%), *M. thermalis* MYTH^T (86.7%) and the acid-tolerant type I methanotrophic strain M200 (87.3%; Supplementary Table S3). Furthermore, partial PmoA based trees, Neighbor-Joining tree (**Figure 4**), Maximum-Likelihood (Supplementary Figure S3), and Minimum-Evolution (Supplementary Figure S5), showed similar topology. It suggested that the isolates were well-separated from other taxonomically described representatives of type I methanotrophs.

BLASTp search of amino acid sequences of MxaF protein from strains BFH1 and BFH2, showed highest identity to *Methylocaldum* sp. 5FB and *Methylocaldum* sp. E10a (96–96.6%), *M. szegediense* (94%), *M. capsulatus* (93.4%), *Methyloparacoccus murrellii* strains R-49797^T and OS501^T (92.2–92.6%), *Methylomarinum vadi* IT-4^T and *Methylovulum miyakonense* HT12^T (88.5%) and *Methyloglobus morosus* Kom1^T (87.8%; Supplementary Table S4). CbbL sequences of strains BFH1 and BFH2 showed closest relationship to the methanotrophic bacteria *M. capsulatus* strain Bath (GenBank Accession No. AF447860) and *M. szegediense* (GenBank Accession No. WP_026609010) with 98.1 and 95.0% amino acid sequence identity, respectively.

DISCUSSION

Through cultivation efforts, two novel moderately thermophilic and acid-tolerant methane oxidizers, designated BFH1 and BFH2, were isolated from a methane-rich slightly acidic tropical topsoil habitat. The isolates were strictly aerobic and showed methylotrophic growth with methane and methanol as sole carbon and energy source. They showed optimal growth on LMA medium, which was 10 times more diluted than AMS medium and supplemented with NH_4Cl.

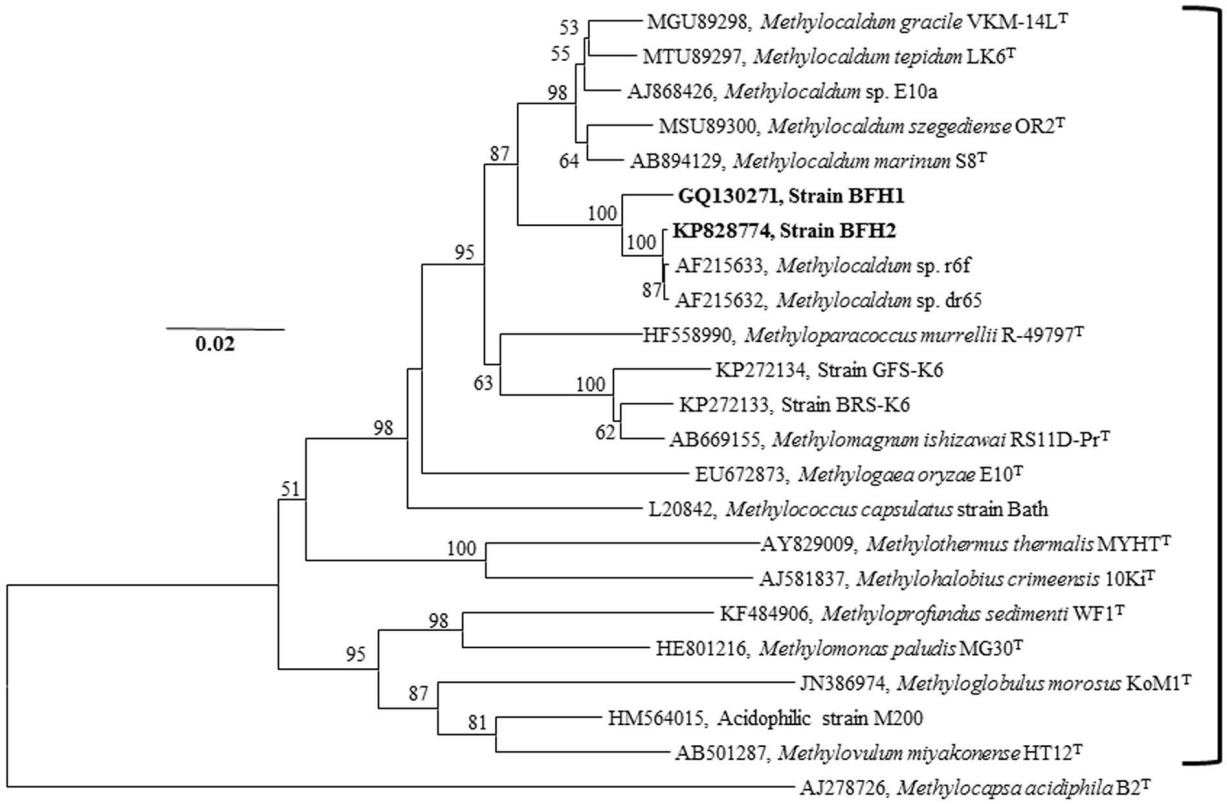

FIGURE 3 | 16S rRNA gene-based Neighbor-Joining tree showing the phylogenetic placement of strains BFH1 and BFH2 (indicated in bold) among described gammaproteobacterial MOB. The tree was constructed using MEGA6 software package. Bootstrap values are based on 1000 resamplings. Bar, 0.02 substitutions per nucleotide position. The type II alphaproteobacterial methanotroph *Methylocapsa acidiphila* (AJ278726) was used as an outgroup. GenBank accession numbers are given in front of the respective isolates name. The evolutionary distances were computed using the Kimura 2-parameter method and there were a total of 1200 positions in the final dataset. Bootstrap values less than 50% are not shown.

Phylogenetic analysis of the 16S rRNA gene of the isolates revealed that they may represent a new clade within the family *Methylococcaceae* of the class *Gammaproteobacteria*, and this phylogenetic inference was supported by the physiological properties and chemotaxonomic analyses (**Tables 1** and **2**). The strain BFH1 differed significantly from characterized *Methylocaldum* spp. in major PLFA compositions, genomic DNA mol% G+C content (>4% difference), pH growth range (4.2–7.5 versus 6–8), and growth on methanol. The isolates are moderately thermophilic, not thermotolerant or true thermophilic, as they cannot grow above 65°C. Recently, a moderately thermoacidophilic methanotroph of the family *Methylothermaceae*, *Methylothermus subterraneus* HTM55^T, was validly described and this bacterium is able to grow at temperature up to 65°C and at pH 5.2–7.5 (Hirayama et al., 2011). Three moderately acidophilic strains *M. paludis* MG30, M200 and M5, were the first isolates of type I methanotrophs that were recovered from an acidic wetland and *Sphagnum* peat bog, respectively (Kip et al., 2011; Danilova et al., 2013). These strains are psychrotolerant or mesophilic methanotrophs (temperature range 4–30°C), and able to grow between pH 3.8 and 7.3. Strain M200 is most closely related to the genus *Methylovulum* and probably represent a novel genus, whereas strains M5 and *M. paludis* MG30 are affiliated with the genus *Methylomonas*. However, these strains have been isolated from temperate environments. A number of type I methanotrophs of the family *Methylococcaceae* (genera: *Methylogaea* and *Methylomagnum*; strains: BRS-K6 and GFS-K6) from different tropical habitats (rice paddy fields and methane seep pond sediments) have been described. They are mesophilic (growth temperature range 8–37°C), neutrophilic (pH range 5–9) and non-thermotolerant (Geymonat et al., 2011; Islam et al., 2015; Khalifa et al., 2015). However, isolates BFH1 and BFH2 showed typical moderately thermophilic features (temperature range 30–60°C), and they were growing at pH values between 4.2 and 7, which indicates that they are most probably acid-tolerant or slightly acidophilic methanotrophs. This may reflect the isolates' adaptation to the *in situ* pH and temperature of the tropical habitat they were derived from. Furthermore, pairwise distance analysis of the 16S rRNA gene showed a relatively high sequence identity (>97.6%) with the thermophilic methanotrophic isolates, *Methylocaldum* sp. dr65 and *Methylocaldum* sp. r6f, from landfill cover soils. The high sequence identity of these isolates to strains BFH1 and BFH2 suggests that they may have common physiology and metabolism properties.

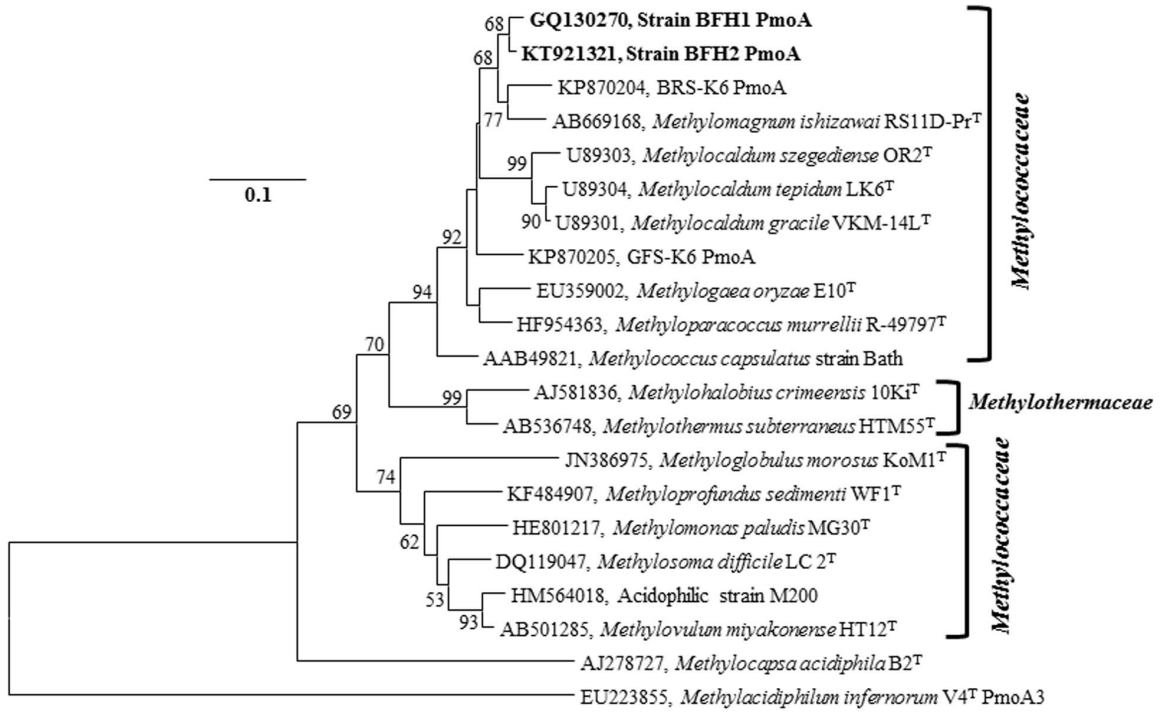

FIGURE 4 | PmoA Neighbor-Joining tree (based on predicted protein sequences) of the strains BFH1 and BFH2 related to other Gammaproteobacterial methane oxidizing bacteria. GenBank accession numbers are given in front of the respective isolates name. Bootstrap values (1000 replicates) less than 50% are not shown. Bar, 0.1 substitutions per nucleotide position. The evolutionary distances were computed using the JTT matrix-based method and there were a total of 140 positions in the final dataset. The type II Alphaproteobacterial methanotroph *M. acidiphila* (AJ278727) and a thermoacidophilic verrucomicrobial methanotroph, *Methylacidiphilum infernorum* V4 PmoA3 (EU223855), were used as an outgroup. Evolutionary analyses were conducted in MEGA6.

Growth of the isolates was inhibited by acetylene, indicating that functional methane oxidation enzymes were present, whereas the naphthalene oxidation assay was negative, which verified the absence of soluble methane monooxygenase. These results are in accordance with those from other isolates of thermophilic or thermoacidophilic methanotrophs (Dedysh, 2009; Op den Camp et al., 2009). The genes *pmoA* and *mxaF* that encode key methane metabolism enzymes have been used as phylogenetic markers for both alphaproteobacterial and gammaproteobacterial methanotrophs. Detection of these genes in the isolates suggests that they produce these key methane oxidation enzymes. In addition, analyses of *mmoX* and Southern hybridization results indicate that the isolates do not have genes encoding the soluble methane monooxygenase. This gene is generally found in mesophilic and thermotolerant MOB, but not in moderately thermophilic or thermophilic MOB.

The major fatty acid in strain BFH was C16:0. This fatty acid is a major component in gammaproteobacterial thermophilic and thermotolerant (genera: *Methylocaldum*, *Methylothermus*, *Methylococcus*) methanotrophs (22–65%), whereas mesophilic and psychrotolerant type I methanotrophs (genera: *Methylomonas, Methylobacter, Methylomicrobium, Methylosoma, Methylosphaera, Methylosarcina*) contain relatively low C16:0 concentrations (4–18%; Bowman et al., 1997; Rahalkar et al., 2007; Kip et al., 2011). Exceptions are the mesophilic genus *Methylovulum* (47%) of type I methanotrophs (Iguchi et al., 2011), and the mesophilic genus *Methylohalobius* (23%; Heyer et al., 2005). The unsaturated fatty acid, C16:1ω7c was also found in strain BFH1. This fatty acid was not reported in the thermotolerant and moderately thermophilic genus *Methylocaldum*, whereas the thermotolerant genus *Methylococcus* contained about 10–23%, and the thermophilic genus *Methylothermus* had even lesser amounts (2–3.5%) of this unsaturated fatty acid (**Table 2**). On the other hand and interestingly, high amounts of this fatty acid (12–60%) are usually found in cold-adapted type I methanotrophs (Rahalkar et al., 2007; Kip et al., 2011; Deutzmann et al., 2014). Strain BFH1 also contained relatively high amounts of C17:0*cyc* (cyclopropane) as compared to other thermotolerant and thermophilic MOB. This makes its fatty acid composition unique, which can be used as a diagnostic feature, differentiating it from other methanotrophs.

In the present work, isolation of novel aerobic methanotrophs from methane-rich tropical soil has been achieved. No thermoacidophilic or acid-tolerant moderately thermophilic MOB has been described previously in the order *Methylococcales*. To the best of our knowledge, strains BFH1 and BFH2 are the first acid-tolerant moderately thermophilic methanotrophs of the class *Gammaproteobacteria* to be isolated. Therefore, the description of these strains will increase our knowledge of this ecophysiological group. The present study suggests that methanotrophs such as strains BFH1 and BFH2 may play a vital ecological role in methane-rich moderately acidic

tropical habitats. They are obligate methylotrophs because they can only grow on methane or methanol. Together with *Methylocaldum* sp. dr65 and *Methylocaldum* sp. r6f they may represent one or more new species in a novel genus in the family *Methylococcaceae*. The new clade is probably diverse and widespread, particularly in tropical topsoil habitats. Future whole genome sequencing of the isolates together with biochemical and physiological analyses are expected to afford important insights into their ecophysiology and adaptation. The isolates are possibly coupled to the biogeochemically related reactions, and may provide evidences for controlling methane emissions to the atmosphere from the tropical surface gas blowout areas as well as evolutionary significance. The 16S rRNA, *pmoA, mxaF,* and *cbbL* sequences may assist for further identification of related methanotrophs from various habitats and show how these microorganisms are widely distributed. However, this study may improve our understanding of the methane oxidation process in such methane-rich tropical ecosystems, and extend our knowledge mainly based on studies of methanotrophs from temperate environments, which for instance indicate that enzymes involved in methane oxidation have average optimum temperature at 25°C (Aronson et al., 2013).

AUTHOR CONTRIBUTIONS

TI, VT, ØL, LB, LØ, and N-KB designed the experiments, analyzed the data, and wrote the manuscript. TI collected samples and isolated pure cultures and performed experiments. LØ and N-KB contributed reagents/materials/TEM analysis. VT has revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00851

REFERENCES

Alam, M. S., and Jia, Z. (2012). Inhibition of methane oxidation by nitrogenous fertilizers in a paddy soil. *Front. Microbiol.* 3:246. doi: 10.3389/fmicb.2012.00246

Arai, H., Hadi, A., Darung, U., Limin, S. H., Hatano, R., and Inubushi, K. (2014). A methanotrophic community in a tropical peatland is unaffected by drainage and forest fires in a tropical peat soil. *Soil Sci. Plant Nutri.* 60, 577–585. doi: 10.1080/00380768.2014.922034

Aronson, E. L., Allison, S. D., and Helliker, B. R. (2013). Environmental impacts on the diversity of methane-cycling microbes and their resultant function. *Front. Microbiol.* 4:225. doi: 10.3389/fmicb.2013.00225

Baxter, N. J., Hirt, R. P., Bodrossy, L., Kovacs, K. L., Embley, T. M., Prosser, J. I., et al. (2002). The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Arch. Microbiol.* 177, 279–289. doi: 10.1007/s00203-001-0387-x

Bodrossy, L., Holmes, E. M., Holmes, A. J., Kovacs, K. L., and Murrell, J. C. (1997). Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, *Methylocaldum* gen. nov. *Arch. Microbiol.* 168, 493–503. doi: 10.1007/s002030050527

Bodrossy, L., Kovács, K. L., McDonald, I. R., and Murrell, J. C. (1999). A novel thermophilic methane-oxidising γ-Proteobacterium. *FEMS Microbiol. Lett.* 170, 335–341. doi: 10.1111/j.1574-6968.1999.tb13392.x

Bowman, J. P., McCammon, S. A., and Skerrat, J. H. (1997). *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. *Microbiology* 143, 1451–1459. doi: 10.1099/00221287-143-4-1451

Bowman, J. P., Sly, L. I., Nichols, P. D., and Hayward, A. C. (1993). Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Syst. Evol. Microbiol.* 43, 735–753.

Danilova, O. V., Kulichevskaya, I. S., Rozova, O. N., Detkova, E. N., Bodelier, P. L., and Trotsenko, Y. A. (2013). *Methylomonas paludis* sp. *nov.*, the first acid-tolerant member of the genus *Methylomonas*, from an acidic wetland. *Int. J. Syst. Evol. Microbiol* 63, 2282–2289. doi: 10.1099/ijs.0.045658-0

Dedysh, S. (2009). Exploring methanotroph diversity in acidic northern wetlands: molecular and cultivation-based studies. *Microbiology* 78, 655–669. doi: 10.1134/S0026261709060010

Dedysh, S. N., Belova, S. E., Bodelier, P. L., Smirnova, K. V., Khmelenina, V. N., Chidthaisong, A., et al. (2007). *Methylocystis heyeri* sp. nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. *Int. J. Syst. Evol. Microbiol.* 57, 472–479. doi: 10.1099/ijs.0.64623-0

Deutzmann, J. S., Hoppert, M., and Schink, B. (2014). Characterization and phylogeny of a novel methanotroph, *Methyloglobulus morosus* gen. nov., spec. nov. *Syst. Appl. Microbiol.* 37, 165–169. doi: 10.1016/j.syapm.2014.02.001

Dianou, D., Ueno, C., Ogiso, T., Kimura, M., and Asakawa, S. (2012). Diversity of cultivable methane-oxidizing bacteria in microsites of a rice paddy field: investigation by cultivation method and fluorescence in situ hybridization (FISH). *Microbes and Environments.* 27, 278–287. doi: 10.1264/jsme2.ME11327

Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S., et al. (2007). Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. *Nature* 450, 879–882. doi: 10.1038/nature06411

Eshinimaev, B., Medvedkova, K. A., Khmelenina, V. N., Suzina, N. E., Osipov, G. A., and Lysenko, A. M. (2004). New thermophilic methanotrophs of the genus *Methylocaldum*. *Mikrobiologiia* 73, 530–539.

Etiope, G., and Klusman, R. W. (2002). Geologic emissions of methane to the atmosphere. *Chemosphere* 49, 777–789. doi: 10.1016/S0045-6535(02)00380-6

Foster, J. W., and Davis, R. H. (1966). A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. *J. Bacteriol.* 91, 1924–1931.

Geymonat, E., Ferrando, L., and Tarlera, S. E. (2011). *Methylogaea oryzae* gen. nov., sp. nov., a mesophilic methanotroph isolated from a rice paddy field. *Int. J. Syst. Evol. Microbiol.* 61, 2568–2572. doi: 10.1099/ijs.0.028274-0

Graham, D., Korich, D., LeBlanc, R., Sinclair, N., and Arnold, R. (1992). Applications of a colorimetric plate assay for soluble methane monooxygenase activity. *Appl. Environ. Microbiol.* 58, 2231–2236.

Hanson, R. S., and Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiol. Rev.* 60, 439–471.

Heyer, J., Berger, U., Hardt, M., and Dunfield, P. F. (2005). *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. *Int. J. Syst. Evol. Microbiol.* 55, 1817–1826. doi: 10.1099/ijs.0.63213-0

Hirayama, H., Abe, M., Miyazaki, M., Nunoura, T., Furushima, Y., Yamamoto, H., et al. (2014). *Methylomarinovum caldicuralii* gen. nov., sp. nov., a moderately thermophilic methanotroph isolated from a shallow submarine hydrothermal system, and proposal of the family *Methylothermaceae* fam. nov. *Int. J. Syst. Evol. Microbiol.* 64, 989–999. doi: 10.1099/ijs.0.058172-0

Hirayama, H., Suzuki, Y., Abe, M., Miyazaki, M., Makita, H., Inagaki, F., et al. (2011). *Methylothermus subterraneus* sp. nov., a moderately thermophilic methanotroph isolated from a terrestrial subsurface hot aquifer. *Int. J. Syst. Evol. Microbiol.* 61, 2646–2653. doi: 10.1099/ijs.0.028092-0

Hoefman, S., van der Ha, D., Iguchi, H., Yurimoto, H., Sakai, Y., and Boon, N. (2014). *Methyloparacoccus murrellii* gen. nov., sp. nov., a methanotroph isolated from pond water. *Int. J. Syst. Evol. Microbiol.* 64, 2100–2107. doi: 10.1099/ijs.0.057760-0

Iguchi, H., Yurimoto, H., and Sakai, Y. (2011). *Methylovulum miyakonense* gen. nov., sp. nov., a type I methanotroph isolated from forest soil. *Int. J. Syst. Evol. Microbiol.* 61, 810–815. doi: 10.1099/ijs.0.019604-0

Islam, T., Jensen, S., Reigstad, L. J., Larsen, O., and Birkeland, N. K. (2008). Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. *Proc. Natl. Acad. Sci. U.S.A.* 105, 300–304. doi: 10.1073/pnas.0704162105

Islam, T., Larsen, Ø., Torsvik, V., Øvreås, L., Panosyan, H., and Murrell, J. C. (2015). Novel methanotrophs of the family *Methylococcaceae* from different geographical regions and habitats. *Microorganisms J.* 3, 484–499. doi: 10.3390/microorganisms3030484

Khalifa, A., Lee, C. G., Ogiso, T., Ueno, C., Dianou, D., Demachi, T., et al. (2015). *Methylomagnum ishizawai* gen. nov., sp. nov., a mesophilic type I methanotroph isolated from rice rhizosphere. *Int. J. Syst. Evol. Microbiol.* 65, 3527–3534. doi: 10.1099/ijsem.0.000451

Khan, M. A. I., and Nasir, F. B. (2014). A Review over major gas blowouts in Bangladesh, their effects and the measures to prevent them in future. *Int. J. Sci Technol. Res.* 3, 109–113.

Kip, N., Ouyang, W., van Winden, J., Raghoebarsing, A., van Niftrik, L., and Pol, A. (2011). Detection, isolation, and characterization of acidophilic methanotrophs from *Sphagnum mosses*. *Appl. Environ. Microbiol.* 77, 5643–5654. doi: 10.1128/AEM.05017-11

Knief, C. (2015). Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on pmoA as molecular marker. *Front. Microbiol.* 6:1346. doi: 10.3389/fmicb.2015.01346

Knief, C., and Dunfield, P. F. (2005). Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. *Environ. Microbiol.* 7, 1307–1317. doi: 10.1111/j.1462-2920.2005.00814.x

Knief, C., Vanitchung, S., Harvey, N. W., Conrad, R., Dunfield, P. F., and Chidthaisong, A. (2005). Diversity of methanotrophic bacteria in tropical upland soils under different land uses. *Appl. Environ. Microbiol.* 71, 3826–3831. doi: 10.1128/AEM.71.7.3826-3831.2005

Lau, E., Fisher, M. C., Steudler, P. A., and Cavanaugh, C. M. (2013). The methanol dehydrogenase gene, mxaF, as a functional and phylogenetic marker for proteobacterial methanotrophs in natural environments. *PLoS ONE* 8:e56993. doi: 10.1371/journal.pone.0056993

McDonald, I. R., Bodrossy, L., Chen, Y., and Murrell, J. C. (2008). Molecular ecology techniques for the study of aerobic methanotrophs. *Appl. Environ. Microbiol.* 74, 1305–1315. doi: 10.1128/AEM.02233-07

McDonald, I. R., and Murrell, J. C. (1997). The methanol dehydrogenase structural gene mxaF and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl. Environ. Microbiol.* 63, 3218–3224.

Nazaries, L., Murrell, J. C., Millard, P., Baggs, L., and Singh, B. K. (2013). Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions. *Environ. Microbiol.* 15, 2395–2417. doi: 10.1111/1462-2920.12149

Op den Camp, H. J., Islam, T., Stott, M. B., Harhangi, H. R., Hynes, A., and Schouten, S. (2009). Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ. Microbiol. Rep.* 1, 293–306. doi: 10.1111/j.1758-2229.2009.00022.x

Pol, A., Heijmans, K., Harhangi, H. R., Tedesco, D., Jetten, M. S., and Op den Camp, H. J. (2007). Methanotrophy below pH 1 by a new *Verrucomicrobia* species. *Nature* 450, 874–878. doi: 10.1038/nature06222

Rahalkar, M., Bussmann, I., and Schink, B. (2007). *Methylosoma difficile* gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral sediment of Lake Constance. *Int. J. Syst. Evol. Microbiol.* 57, 1073–1080. doi: 10.1099/ijs.0.64574-0

Rattanachomsri, U., Kanokratana, P., Eurwilaichitr, L., Igarashi, Y., and Champreda, V. (2011). Culture-independent phylogenetic analysis of the microbial community in industrial sugarcane bagasse feedstock piles. *Biosci. Biotech. Biochem.* 75, 232–239. doi: 10.1271/bbb.100429

Sharp, C. E., Martínez-Lorenzo, A., Brady, A. L., Grasby, S. E., and Dunfield, P. F. (2014a). Methanotrophic bacteria in warm geothermal spring sediments identified using stable-isotope probing. *FEMS Microbiol. Ecol.* 90, 92–102. doi: 10.1111/1574-6941.12375

Sharp, C. E., Smirnova, A. V., Graham, J. M., Stott, M. B., Khadka, R., Moore, T. R., et al. (2014b). Distribution and diversity of *Verrucomicrobia* methanotrophs in geothermal and acidic environments. *Environ. Microbiol.* 16, 1867–1878. doi: 10.1111/1462-2920.12454

Takeuchi, M., Kamagata, Y., Oshima, K., Hanada, S., Tamaki, H., Marumo, K., et al. (2014*). Methylocaldum marinum* sp. nov., a thermotolerant, methane-oxidizing bacterium isolated from marine sediments, and emended description of the genus *Methylocaldum*. *Int. J. Syst. Evol. Microbiol.* 64, 3240–3246. doi: 10.1099/ijs.0.063503-0

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tavormina, P. L., Hatzenpichler, R., McGlynn, S., Chadwick, G., Dawson, K. S., Connon, S. A., et al. (2015). *Methyloprofundus sedimenti* gen. nov., sp. nov., an obligate methanotroph from ocean sediment belonging to the 'deep sea-1' clade of marine methanotrophs. *Int. J. Syst. Evol. Microbiol.* 65, 251–259. doi: 10.1099/ijs.0.062927-0

Tourova, T. P., Kovaleva, O. L., Sorokin, D. Y., and Muyzer, G. (2010). Ribulose-1, 5-bisphosphate carboxylase/oxygenase genes as a functional marker for chemolithoautotrophic halophilic sulfur-oxidizing bacteria in hypersaline habitats. *Microbiology* 156, 2016–2025. doi: 10.1099/mic.0.034603-0

Trotsenko, Y. A., and Murrell, J. C. (2008). Metabolic aspects of aerobic obligate methanotrophy. *Adv. Appl. Microbiol.* 63, 183–229. doi: 10.1016/S0065-2164(07)00005-6

Tsubota, J., Eshinimaev, B., Khmelenina, V. N., and Trotsenko, Y. A. (2005). *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *Int. J. Syst. Evol. Microbiol.* 55, 1877–1884. doi: 10.1099/ijs.0.63691-0

van der Linde, K., Lim, B. T., Rondeel, J. M., Antonissen, L. P., and de Jong, G. M. (1999). Improved bacteriological surveillance of haemodialysis fluids: a comparison between Tryptic soy agar and Reasoner's 2A media. *Nephrol. Dial. Transplant.* 14, 2433–2437. doi: 10.1093/ndt/14.10.2433

van Teeseling, M. C., Pol, A., Harhangi, H. R., van der Zwart, S., Jetten, M. S., and Op den Camp, H. J. (2014). Expanding the verrucomicrobial methanotrophic world: description of three novel species of *Methylacidimicrobium* gen. nov. *Appl. Environ. Microbiol.* 80, 6782–6791. doi: 10.1128/AEM.01838-14

Vorobev, A. V., Baani, M., Doronina, N. V., Brady, A. L., Liesack, W., Dunfield, P. F., et al. (2011). *Methyloferula stellata* gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. *Int. J. Syst. Evol. Microbiol.* 61, 2456–2463. doi: 10.1099/ijs.0.028118-0

Wasmund, K., Kurtböke, D. I., Burns, K. A., and Bourne, D. G. (2009). Microbial diversity in sediments associated with a shallow methane seep in the tropical Timor Sea of Australia reveals a novel aerobic methanotroph diversity. *FEMS Microbiol. Ecol.* 68, 142–151. doi: 10.1111/j.1574-6941.2009.00667.x

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

Whittenbury, R., Phillips, K. C., and Wilkinson, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* 61, 205–218. doi: 10.1099/00221287-61-2-205

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Panamanian frog species host unique skin bacterial communities

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Vertebrates, including amphibians, host diverse symbiotic microbes that contribute to host disease resistance. Globally, and especially in montane tropical systems, many amphibian species are threatened by a chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), that causes a lethal skin disease. Bd therefore may be a strong selective agent on the diversity and function of the microbial communities inhabiting amphibian skin. In Panamá, amphibian population declines and the spread of Bd have been tracked. In 2012, we completed a field survey in Panamá to examine frog skin microbiota in the context of Bd infection. We focused on three frog species and collected two skin swabs per frog from a total of 136 frogs across four sites that varied from west to east in the time since Bd arrival. One swab was used to assess bacterial community structure using 16S rRNA amplicon sequencing and to determine Bd infection status, and one was used to assess metabolite diversity, as the bacterial production of anti-fungal metabolites is an important disease resistance function. The skin microbiota of the three Panamanian frog species differed in OTU (operational taxonomic unit, ∼bacterial species) community composition and metabolite profiles, although the pattern was less strong for the metabolites. Comparisons between frog skin bacterial communities from Panamá and the US suggest broad similarities at the phylum level, but key differences at lower taxonomic levels. In our field survey in Panamá, across all four sites, only 35 individuals (∼26%) were Bd infected. There was no clustering of OTUs or metabolite profiles based on Bd infection status and no clear pattern of west-east changes in OTUs or metabolite profiles across the four sites. Overall, our field survey data suggest that different bacterial communities might be producing broadly similar sets of metabolites across frog hosts and sites. Community structure and function may not be as tightly coupled in these skin symbiont microbial systems as it is in many macro-systems.

Keywords: amphibian, *Batrachochytrium dendrobatidis*, chytrid fungus, Kolmogorov-Smirnov measure, microbiome, microbiota, structure-function relationship

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INTRODUCTION

All animals serve as hosts to symbiotic microorganisms that, along with their genetic contributions, constitute their microbiome. We have long understood that the microbes that reside in the gut are diverse and are important in helping digest food. More recently, we have begun to appreciate the incredible diversity of these symbiotic microbial communities and to recognize that they reside

throughout the body (Cho and Blaser, 2012; Ursell et al., 2012). The composition of these microbial communities can strongly influence many facets of host health and disease resistance (Cho and Blaser, 2012; Fierer et al., 2012; McFall-Ngai et al., 2013). Our knowledge in this area is growing in large part because of advances in molecular microbiology that now allow us to study these complex microbial communities in much more detail. Culture-independent studies, while initially focused on revealing diversity, now clearly indicate complex interactions in these communities both among microbes and among microbes and their host (McFall-Ngai et al., 2013; Boon et al., 2014; Manor et al., 2014).

Recent work on amphibians provides an example of the role that the natural microbiota may have in preventing disease. *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen first isolated and described in 1999 (Longcore et al., 1999), causes the potentially lethal skin disease, chytridiomycosis, in susceptible amphibians. Bd has been associated with many amphibian population declines in recent decades since it was first observed in dead and dying frogs from both Central America and Australia (Berger et al., 1998). The adaptive and innate immune systems, and in particular the production of antimicrobial peptides in amphibian skin, has an important role in preventing Bd infection in some host species (McMahon et al., 2014; Rollins-Smith et al., 2015). However, the skin of healthy amphibians is also host to a diverse resident bacterial community (McKenzie et al., 2012; Walke et al., 2014), and a number of these bacteria can inhibit Bd growth (Harris et al., 2006; Flechas et al., 2012; Bell et al., 2013; Becker et al., 2015b; Woodhams et al., 2015). Inhibition is likely due to bacterially-produced secondary metabolites inhibiting Bd zoospore colonization or development (Brucker et al., 2008a,b; Becker et al., 2009; Lam et al., 2011; Bell et al., 2013). In addition, experiments have demonstrated that a supplemented protective microbiota can reduce morbidity and mortality in some amphibians infected with Bd (Harris et al., 2009), and that reduction of the cutaneous microbial community can worsen disease outcomes (Becker and Harris, 2010). Several recent experimental studies have also linked the structure of the microbial communities on the skin with Bd exposure (Jani and Briggs, 2014; Becker et al., 2015a) and infection outcome (Becker et al., 2015a). These studies illustrate that Bd may affect bacterial skin communities both by causing differential mortality of bacterial species and by selecting for hosts with protective bacterial communities.

Panamá has historically had one of the most diverse assemblages of amphibians in the Neotropics (reviewed in Jaramillo et al., 2010) and is one of the places where Bd was first described (Berger et al., 1998). Since its initial discovery, Bd has been moving eastward across Panamá (Lips et al., 2006; Woodhams et al., 2008; Rebollar et al., 2014), and its movement there has been closely monitored. As it has spread, many Panamanian amphibian populations have been decimated by Bd; at one highland site, 25 of 63 named amphibian species (∼40%) disappeared following the arrival of Bd (Crawford et al., 2010). However, not all amphibian species are susceptible to chytridiomycosis (Smith et al., 2009; Crawford et al., 2010; Kilburn et al., 2010), and even some populations of what were assumed to be Bd-susceptible species in Panamá seem to be persisting at some sites with the fungus (Hertz et al., 2012; Perez et al., 2014). Many hypotheses have been put forward to explain this variation, including differential immune responses among species and variation in disease dynamics due to abiotic factors, such as temperature and humidity, that differ across sites (Blaustein et al., 2011, 2012; Venesky et al., 2014).

To explore the potential role of the skin microbiota in disease resistance in free-living Panamanian frogs, we used a field survey of three frog species across four sites on a west-east gradient in Panamá, with Bd having arrived earlier (∼2006) at the western site and later (∼2011) at the eastern site (Woodhams et al., 2008; Rebollar et al., 2014). We first described the microbiota of each frog host species. We then conducted a broader scale comparison of the microbiota from Panamanian species with that from three frog species from the eastern US, which have a much longer history of coexistence with Bd (Ouellet et al., 2005). Finally, we used the Panamá field survey dataset and the prior knowledge of the distribution of Bd in Panamá to examine the potential link between the structure of bacterial skin symbiont communities and their function in Bd disease resistance. We hypothesized that if Bd is a strong selective force on these symbiont communities, we should see: (1) individuals currently infected with Bd having different microbial community structure (OTUs) and function (metabolite profiles) than non-infected individuals, (2) changes in the microbial communities in terms of alpha- and beta-diversity along this west-east gradient, and less variance in metabolite profiles at sites with a longer history of Bd exposure as selection for anti-fungal production has occurred for a longer period of time, and (3) a stronger correlation between OTU community structure and metabolite production at sites with a longer history of Bd exposure.

MATERIALS AND METHODS

Panamá Field Survey: Sample Collection

In 2012, we sampled 2 or 3 species of frogs at each of four sites in Panamá, for a total of 136 frogs (**Table 1A**). Study sites spanned from central to eastern Panamá (**Figure 1**). Sites, listed from west to east, were: Parque Nacional Altos de Campana (Panamá Province), Parque Nacional Soberanía (Panamá Province), the Mamoní Valley Preserve (Panamá Province), and forest surrounding the community of Nuevo Vigía (Darién Province). The elevation of the ponds and streams where frogs were encountered ranged from 29 to 824 m (**Table 1A**). All animal use was approved by the Institutional Animal Care and Use Committees of Virginia Tech and the Smithsonian Tropical Research Institute, and was completed with permission from the Autoridad Nacional del Ambiente in Panamá.

Two species of treefrogs (Family: Hylidae), *Agalychnis callidryas* (red-eyed treefrogs) and *Dendropsophus ebraccatus* (pantless treefrogs), were sampled at all four sites (*A. callidryas*: $N = 12–20$ individuals/site; *D. ebraccatus*: $N = 9–15$ individuals/site; **Table 1A**). *Agalychnis callidryas* and *D. ebraccatus* are sympatric, breed in ponds and at each of the four sites were sampled at the same pond. One additional

TABLE 1 | Summary of frog species, dates, and sites sampled during field surveys in Panamá (A) and the United States (B) assessing the diversity of bacterial communities on amphibian skin.

Species	Site, province (Panamá) or pond, county/city (US)	Elevation (m)	Sample size (A/J)	SVL (cm) mean, sd	Mass (g) mean, sd
(A) PANAMÁ					
Agalychnis callidryas					
12 Sept 2012	Parque Nacional Altos de Campana, Panamá	824	15 (15/0)	4.7, 0.4	4.3, 1.9
29 Aug 2012	Parque Nacional Soberania, Panamá	50	15 (15/0)	4.8, 0.6	4.7, 2.7
9–11 July 2012	Mamoni Valley Preserve, Panamá	191	20 (20/0)	4.6, 0.5	4.1, 1.7
27 Sept 2012	Nuevo Vigia, Darien	29	12 (12/0)	4.6, 0.5	4.1, 1.6
Craugastor fitzingeri					
30 Aug and 6 Sept 2012	Parque Nacional Soberania, Panamá	64	14 (10/4)	3.5, 1.0	3.9, 2.6
12 July 2012	Mamoni Valley Preserve, Panamá	245	7 (4/3)	2.5, 0.3	1.1, 0.3
Dendropsophus ebraccatus					
12 Sept 2012	Parque Nacional Altos de Campana, Panamá	824	15(15/0)	2.9, 0.2	1.3, 0.4
29 Aug 2012	Parque Nacional Soberania, Panamá	50	14 (14/0)	2.6, 0.3	1.0, 0.5
9–11 July 2012	Mamoni Valley Preserve, Panamá	191	9 (9/0)	2.7, 0.1	1.0, 0.1
25 Sept 2012	Nuevo Vigia, Darien	29	15 (15/0)	2.9, 0.3	1.2, 0.4
(B) UNITED STATES					
Anaxyrus americanus					
20 March 2012	Craig Creek Road, Craig	526	9 (9/0)		
Lithobates catesbeianus					
4 April 2012	Station Pond, Giles	1176	9 (2/7)		
2 May 2012	Pandapas Pond, Montgomery	683	9 (3/6)		
2 April 2012	Peaks View Park, Lynchburg City	200	8 (5/3)		
1 April 2012	Reedy Creek, Richmond City	23	9 (0/9)		
Pseudacris crucifer					
4 April 2012	Sylvatica Pond, Giles	1181	9 (9/0)		
8 March 2012	Pandapas Pond, Montgomery	683	9 (9/0)		
2 April 2012	Food Lion, Campbell	269	7 (7/0)		
1 April 2012	James River Wetland, Richmond City	51	9 (9/0)		

Site elevation and sample characteristics are also summarized, as available, including sample size [total number of individuals as well as the number of adults (A), and juveniles (J)]; snout vent length in centimeters (SVL; mean and standard deviation of all individuals); and mass in grams (mean and standard deviation of all individuals).

species, *Craugastor fitzingeri* (Fitzinger's robber frogs, Family: Craugastoridae), was only encountered, and thus sampled, at two of the four sites ($N = 7$ and 14 individuals/site; **Table 1A**). *Craugastor fitzingeri* was found along the margins of a stream at both sites where this species was sampled.

We sampled the amphibians' skin bacterial community diversity and metabolite profiles by swabbing the surface of the skin, and we determined if individuals were infected with the fungal pathogen, *B. dendrobatidis* (methods and individual Bd results are reported in Rebollar et al., 2014). To sample frogs, we captured them by hand, using new nitrile gloves for each individual. Individuals that could not be swabbed immediately were placed in sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA) for a maximum of 30 min prior to swabbing. Before swabbing, we rinsed each frog by pouring ∼50 ml of sterile deionized water over its body to remove any dirt and transient bacteria (Walke et al., 2014). Each individual was swabbed twice: first, to sample the cutaneous bacterial community and second, to sample metabolites. We sampled the cutaneous bacterial community using sterile rayon swabs (MW113, Medical Wire Equipment & Co. Ltd., Corsham, UK). We then used polyurethane-tipped swabs to assess metabolite profiles (14-960-3J, Fisher Scientific). Prior to use, all polyurethane swabs were pre-treated to remove methanol-soluble impurities by twice rinsing the swab in methanol and then allowing it to dry fully under sterile conditions in the laboratory (Umile et al., 2014). To standardize swabbing across individuals, we swabbed the ventral surface 20 times, each thigh 5 times, and each hind foot 5 times for a total of 40 strokes/swab type/individual. We recorded each individual's life stage (adult or juvenile), snout vent length, and mass before releasing the animal at the site of capture (summarized in **Table 1A**). We placed all swabs in sterile, empty 1.5 mL microcentrifuge tubes and stored them on ice or liquid nitrogen in the field. The swabs were then transferred them to a −80°C freezer in the laboratory until further processing.

Panamá Field Survey: Bacterial Community Structure

Relative abundance of OTUs based on 16S rRNA gene amplicon sequencing was used to assess bacterial community structure on the skin. DNA from the bacterial sampling swabs was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia CA,

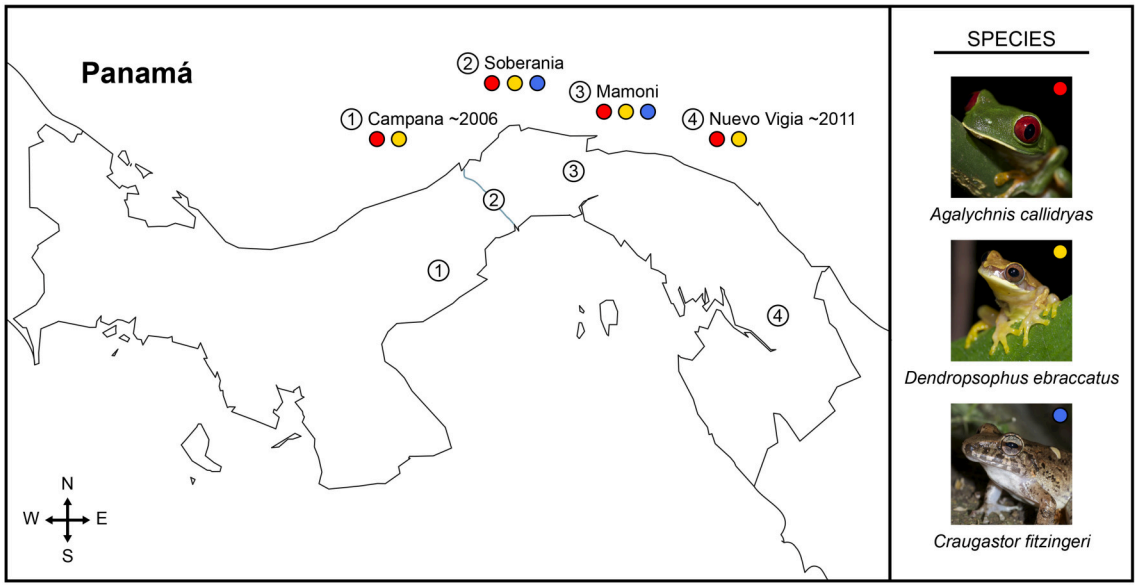

FIGURE 1 | Map of Panamá showing sampled field sites (numbered circles) and the frog species sampled at each location (colored circles: red, *Agalychnis callidryas*; gold, *Dendropsophus ebraccatus*; blue, *Craugastor fitzingeri*) during a field survey assessing the diversity of bacterial communities on amphibian skin. The three species of amphibians we sampled have persisted with the fungal pathogen, *Batrachochytrium dendrobatidis*, for different lengths of time across the four sampled sites, numbered one to four from west to east (i.e., longest at Cerro Campana and shortest at Nuevo Vigia; Woodhams et al., 2008; Rebollar et al., 2014).

USA, Cat. 69506) according to manufacturer's protocol and including an initial lysozyme incubation step for 1 h at 37°C. DNA was eluted in a final volume of 100 µl, and was then used as template to detect Bd infection intensity and prevalence through qPCR as previously reported (Rebollar et al., 2014). In parallel, another portion of the DNA was used as a template for amplicon sequencing using barcoded primers targeting the V4 region of the 16S rRNA gene with primers 515F and 806R (Caporaso et al., 2012). The reverse PCR primer was barcoded with a 12-base error-correcting Golay code to allow for multiplexing of samples, and also contained sequencing adapter regions. PCR reactions contained 13 µL molecular grade PCR water, 10 µL 5 Prime Hot Master Mix, 0.5 µL each of the forward and reverse primers (10 µM final concentration), and 1.0 µL genomic DNA. PCR conditions were: denaturation step 3 min at 94°C, amplification step for 35 cycles for 45 s at 94°C, annealing for 60 s at 50°C, extension 90 s at 72°C, and a final extension of 10 min at 72°C. PCR was done in triplicate, pooled, checked for integrity in a 1% agarose gel and quantified in a QuantiFluor-ST using a florescent dye specific for dsDNA (Promega, Madison WI, USA, Cat. E6090 and Cat. E2670). Each set of 66 samples was pooled by adding 300 ng of DNA from each set of PCR products. Each pooled sample was then cleaned using the QIAquick PCR purification kit (Qiagen, Valencia CA, USA, Cat. 28104). The pooled samples were sent for 250PE Illumina amplicon sequencing at the Dana Farber Cancer Research Institute at Harvard University, Boston, MA.

Raw Illumina 16S rRNA amplicon data files were joined using Fastq-join v. 0.1 (Aronesty, 2011). Joined sequences (length restricted to 250–256 bp) were then processed and quality filtered using the QIIME pipeline (Caporaso et al., 2010b). We used the default settings for demultiplexing with the following exceptions: we allowed for no errors in the barcode, we increased the maximum number of consecutive low quality base calls allowed before truncating a read (r) to 10, and we decreased the minimum number of consecutive high quality base calls to include a read (p) to 0.5. Sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence similarity using the UCLUST method (Edgar, 2010). To represent each OTU, we used the most abundant sequence from each cluster. Representative sequences were aligned to the Greengenes 13_5 reference database (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a). Taxonomy was assigned using the RDP classifier (Wang et al., 2007). We removed OTUs with fewer than 0.001% of the total number of reads, which in this dataset was those with fewer than 112 reads (Bokulich et al., 2013). Sequencing depth per sample ranged from 7067 to 211,411 reads, so we rarefied all samples to a sequencing depth of 7000. Two samples for which there was no corresponding metabolite data were also excluded. The final dataset consisted of 3492 OTUs across 136 samples (62 from *A. callidryas*, 53 from *D. ebraccatus*, and 21 from *C. fitzingeri*). 16S rRNA amplicon sequences were deposited in the NCBI Sequence Read Archive (SRA study accession number: SRP062596).

Panamá Field Survey: Metabolite Profiles

Metabolite profiles were considered a "fingerprint" of the metabolites present on the skin of each frog. Our methods

focused on non-polar, small (<600 m/z) metabolites that are likely to be produced by bacteria, as opposed to host compounds, such as anti-microbial peptides. We do not know which metabolites that comprise the profile are inhibitory to Bd, but we expect the presence of Bd to select for bacteria that produce metabolites that are inhibitory. For metabolite profile analysis, swabs were shipped frozen to Villanova University. To extract metabolites, 1.0 mL of HPLC-grade methanol was added to each swab in its tube. The tubes were capped and vortexed for 5 s, allowed to sit for 10 min, and then vortexed a second time. The swab tip was then removed using forceps. The methanolic extract was slowly filtered into another centrifuge tube using 13 mm syringe filters (0.2 μm PTFE membrane, VWR) to remove any insoluble environmental material. Before use, syringes (1 mL HSW Norm-Ject® disposable syringe) and filters were pre-washed by taking up 1 mL of methanol in the syringe and slowly passing it through the filter. Filtered extracts were evaporated *in vacuo* using a DNA120 SpeedVac with the heating function turned off.

Dried metabolite extracts were reconstituted in 100 μL of methanol containing 1 ppm naphthalene (as internal standard). The reconstituted extracts were analyzed by reversed-phase, high performance liquid chromatography (HPLC, 25 μL injection) using a Shimadzu LC-20 liquid chromatograph equipped with an ACE C18 column (3 μm, 150 × 4.6 mm), a Shimadzu SPD-M20A diode array detector, and an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer (operating in positive electrospray ionization mode). Compounds were separated with a binary mobile phase flowing at 0.5 mL min^{-1} consisting of acidified water (0.1% formic acid, *v/v*; Solvent A) and acidified acetonitrile (0.1% formic acid, *v/v*; Solvent B). The gradient was as follows: 10% B (2 min hold) ramped to a final mobile phase concentration of 100% B over 18 min (5 min hold). Total Wavelength Chromatograms (TWC) of field samples were compared against the TWC of extracted, unused and washed swabs (controls) and also blank, methanol injections. These methods best detect small hydrophobic molecules, such as alkaloids, and therefore do not capture larger and/or more polar molecules, such as antimicrobial peptides.

The retention times of all detected compounds (peaks) were normalized to that of the naphthalene internal standard (20.69 min). The retention time of each chromatographic feature was determined by manually integrating each peak in the TWC using Applied Biosystems Analyst software V.1.5.1. This data set was further manually revised to reduce its size by accounting for slight variations in retention time across multiple samples and focusing on major chemical components. First, compounds that eluted with retention times ± 0.03 min across all samples were investigated for UV-Vis chromophores (λ_{max}) and positively-charged ions. Those compounds with both similar retention times and identical spectroscopic features were pooled and assigned as a single compound. Next, unique compounds only detected in a single sample were disregarded as noise. Finally, all features that had a peak area of less than 3000 mAU × min were disregarded as minor components.

Comparison of the Diversity of Skin Microbial Communities of Amphibians from Panamá and the US

We compared the diversity of the skin bacterial communities of the three species from Panamá to three species from the US that co-occur with Bd: *Lithobates catesbeianus* (American bullfrogs, Family: Ranidae), *Pseudacris crucifer* (spring peepers, Family: Hylidae), and *Anaxyrus americanus* (American toads, Family: Bufonidae). *L. catesbeianus* occupies aquatic habitat and typically breeds in permanent water bodies. Both *P. crucifer* and *A. americanus* occupy terrestrial habitat as adults but breed in a variety of aquatic habitats ranging from permanent to ephemeral. The three US species were sampled between March-May 2012 at several ponds in Virginia, USA, using the same methods as the present study (**Table 1B**; Walke et al., 2015; Belden et al., unpublished data). From Panamá, we included all four populations of *A. callidryas* and *D. ebraccatus*, as well as the two populations of *C. fitzingeri*. From the US, we included four populations of *L. catesbeianus* and *P. crucifer*, and one population of *A. americanus*. To standardize sample sizes, if more than nine individuals were sampled, we randomly chose nine individuals from each population to include in the analysis. Three populations had fewer than nine individuals: one *C. fitzingeri* population ($N = 7$), one *P. crucifer* population ($N = 7$), and one *L. catesbeianus* population ($N = 8$).

Demultiplexed sequences for all individuals from Panamá and the US were combined into a single.fasta file for OTU assignment, taxonomy assignment, and quality filtering. We used the same methods as above, except, for this dataset, we (a) removed OTUs with fewer than 289 reads based on the recommended 0.001% cutoff (Bokulich et al., 2013), and (b) all samples were rarefied to a sequencing depth of 10,000 sequences/sample.

Statistical Analysis

Overview of Statistical Approach

We first completed a general descriptive analysis of our field survey data. This consisted of general summaries of OTU and metabolite diversity of the three host species we studied. In addition, as we found species level differences in OTUs, we assessed what OTUs were responsible for those differences using a novel analysis, the Kolmogorov-Smirnov (K-S) Measure. We also compared alpha- and beta-diversity of bacterial communities from the three Panamanian frog species with samples from three frog species in Virginia, USA, which have likely had a much longer history with Bd (Ouellet et al., 2005). As there were clear differences between frogs from the two regions, we identified some of the key OTUs driving those differences. Then, we used our field survey dataset to assess our specific hypotheses regarding the structure (OTUs) and function (metabolites) of the skin communities on individuals with and without Bd and across the west-east gradient of sites in Panamá that varied in the length of time since Bd arrival. All analyses were run in R (R Core Team, 2014).

OTU and Metabolite Diversity on Amphibians from Panamá

We tested for differences in alpha-diversity of OTUs across species using richness, estimated as the number of unique OTUs/individual, and Faith's phylogenetic diversity. We evaluated community structure in terms of dominance/evenness using the Simpson index (Haegeman et al., 2013). The Simpson index varies between 0 and 1, with values closer to 0 indicating a more even community and values closer to 1 indicating a community dominated by fewer species. We also tested for differences in metabolite richness, estimated as the number of unique metabolites/individual, across species. OTU richness, OTU phylogenetic diversity, and metabolite richness were fit using linear mixed models (package lme4, Bates et al., 2014). The Simpson Index was fit using mixed effect beta regression models (package glmmADMB; Fournier et al., 2012; Skaug et al., 2014). For linear and generalized linear mixed models, we included "site" as a random effect to account for nestedness of samples from the same site. We report "adjusted means" for alpha-diversity measures in the results section that account for the random effects. To estimate P values of factors (e.g., species), we used likelihood ratio tests to compare nested models of increasing simplicity (Zuur et al., 2009). If analyses indicated significant differences among species, we conducted pairwise comparisons to determine which species were similar or different from one another. For linear mixed models, we used Tukey's Honest Significant Different tests to correct for multiple comparisons (function ghlt, package multcomp; Hothorn et al., 2008). This option was not available for the mixed effect beta regression models. Therefore, to determine which species were similar or different from one another, we reran the model on subsets of the dataset representing each pairwise species comparison. For analyses here and below, OTU richness and phylogenetic diversity were log-transformed to better meet assumptions of normality.

For beta-diversity, we tested whether variation in OTU community structure and metabolite profiles could be explained by species using permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) using the function adonis in the vegan package (Oksanen et al., 2013). Separate tests were run for OTUs (relative abundance) and metabolite profiles (presence/absence), and in each case we accounted for the sampling site in the models using the "strata" argument in the adonis function in R. Metabolite profiles were assessed based on presence/absence because the nature of our HPLC-MS method did not allow us to determine relative abundance of the different metabolites on individual frogs. Community data were transformed to distance matrices based on Bray–Curtis and Jaccard dissimilarities for the OTU and metabolite datasets, respectively. If species effects were significant, we conducted pairwise comparisons using PERMANOVA to determine which species were different from one another. To visualize these results, we used nonmetric multi-dimensional scaling (NMDS).

To identify the OTUs driving the variation among the three frog species ($K = 3$), we used a variable screening technique called the K-S measure (Loftus et al., 2015). As discussed in Loftus et al. (2015), other approaches are available to isolate important OTUs, including Sure Information Screening (Fan and Lv, 2008), LASSO (Tibshirani, 1996), and Indicator Species Analyses (Da Caceres et al., 2010), however such methods rely on strong analytical assumptions and/or do not apply directly to a multinomial response. Thus, to relax analytical assumptions and assess the relationship between OTUs and frog species (a multinomial response), we use the K-S measure. The K-S measure is an extension of the K-S test statistic (Kolmogorov, 1933; Smirnov, 1936). The original K-S test statistic assesses the difference (or lack thereof) in $K = 2$ distributions based on the largest absolute distance between empirical distribution functions (e.g., **Figures 2A,B**); an empirical distribution function is an observed cumulative distribution function. As an extension of the K-S statistic, the K-S measure simultaneously assesses differences in $K > 2$ distributions, using the weighted sum of the K-S statistics for all pairwise comparisons of distributions defined by K groups. The weights are proportional to the number of observations used to calculate each K-S statistic. In this case, to determine the OTUs driving the variation between our $K = 3$ Panamanian frog species, the K-S measure for each OTU was calculated as the weighted sum of the three (3 choose 2) K-S test statistics: (1) comparing *A. callidryas* to *D. ebraccatus*, (2) comparing *A. callidryas* to *C. fitzingeri*, and (3) comparing *D. ebraccatus* to *C. fitzingeri*.

The K-S measure ranges from 0 to 1, where values closer to one imply a greater difference between the K distributions than values closer to zero. K-S measures were calculated for each OTU in our field survey dataset, and the values were plotted in descending order (**Figure 2C**). We used a natural break in the K-S measures detected after the 46th value as an initial cutoff for OTUs to retain (Loftus et al., 2015). This subset of OTUs was then placed into a multinomial regression model in which species was the response and the selected OTUs were the covariates, and we eliminated an additional 5 of the 46 OTUs that were not significant at a level of $P = 0.05$ for any level of the response. To evaluate how well the final subset of 41 OTUs identified the three species, we used a holdout cross-validation procedure, working with a training sample of 68 randomly chosen frogs, which left a holdout testing sample size of 68 frogs. Of the 68 frogs in the holdout set, we found that the subset of 41 OTUs correctly classified 27/31 *A. callidryas*, 10/10 *C. fitzingeri*, and 20/27 *D. ebraccatus*.

Comparison of the Diversity of Skin Microbial Communities of Amphibians from Panamá and the US

We tested for differences in alpha diversity across zones (temperate or tropical) using linear and mixed effect beta regression models as above (linear models for richness and phylogenetic diversity; mixed effect beta regression for Simpson Index). However, when testing for differences between zones, we included a random effect of "site" nested within "species" to account for nestedness of samples of the same species from the same sites.

We assessed if variation in beta diversity was explained by zone using PERMANOVA. We also tested for homogeneity of group dispersions. Community data were converted to distance matrices based on Bray–Curtis dissimilarities. Beta diversity was

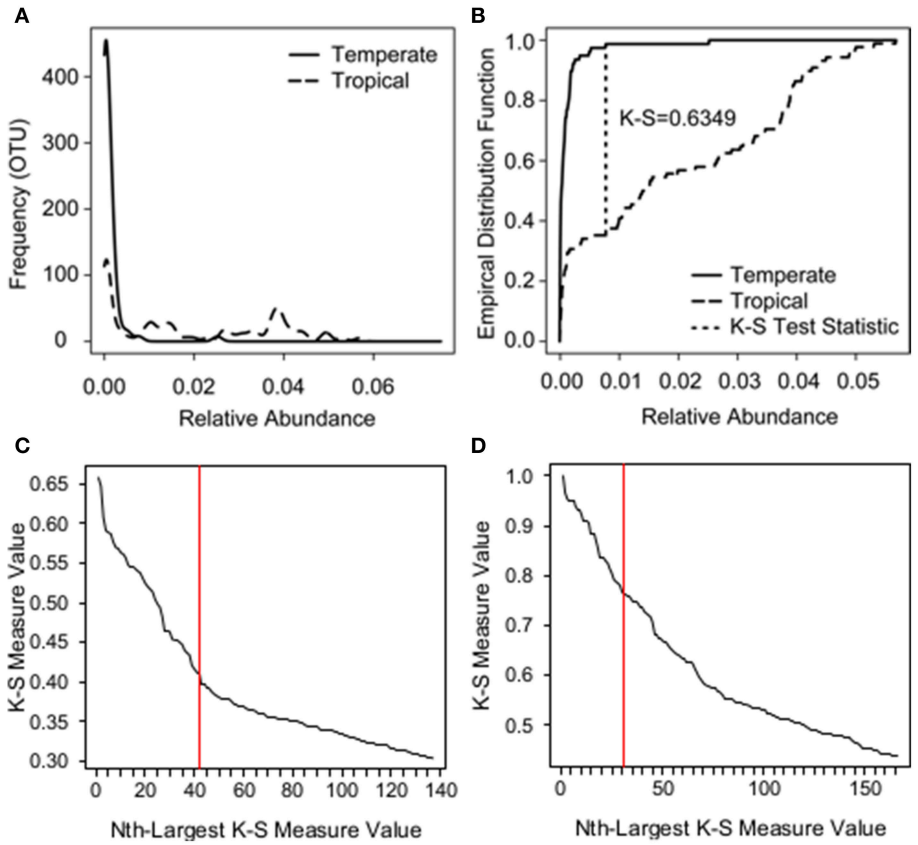

FIGURE 2 | Example of how the K-S test statistic is determined and K-S measures plotted in descending order for species and zone analyses. (A) The relative abundance distributions of a single bacterial OTU across two groups: temperate amphibians (solid line) vs. tropical amphibians (long dash). **(B)** The K-S test statistic assesses the difference between the two distributions based on the largest absolute distance between empirical distribution functions (short dash). The empirical distribution function is an observed cumulative distribution function, where, in this case, the y-axis represents the proportion of observations less than or equal to a given relative abundance on the x-axis. **(C)** K-S measures for each OTU plotted in descending order for species analysis. **(D)** K-S measures for each OTU plotted in descending order for zone analysis.

visualized using NMDS. We then used the K-S measure as described above to determine OTUs that distinguished tropical skin microbiomes from temperate ones. OTUs were sorted in descending order by their K-S measures (**Figure 2D**), which in this case were equivalent to the K-S statistics because there were only two groups ($K = 2$). We determined a natural cutoff after 31 OTUs. This subset of 31 OTUs perfectly split the tropical and temperate samples when plotted using NMDS (using Bray–Curtis dissimilarities). We explored reducing this number further through multinomial regression, but three of the 31 OTUs only appeared in one of the two zones. This posed a problem for further analysis; a logistic regression model is unidentifiable when including these three OTUs.

Impact of Bd on Amphibian Microbiome Structure and Function: Hypothesis Tests

Bd infection status

We hypothesized that on individual frogs actively infected with Bd, we would see shifts in the structure and function of the skin microbiome relative to uninfected individuals. To assess if Bd infection status was associated with changes in alpha diversity, we tested for a difference in OTU richness, OTU phylogenetic diversity, and metabolite richness across Bd infected and uninfected individuals using linear mixed models, including a random effect of "site" nested within "species" to account for nestedness of samples of the same species from the same site. To assess whether Bd infection status (infected or not) was associated with changes in OTU community structure or metabolite profiles on individuals, we used PERMANOVA, including a "species × site" term with the strata argument in the adonis model to account for species and site level effects. To assess whether Bd infection decreased variation in community structure or metabolite profiles (i.e., there is strong selection for anti-fungal metabolite production and the taxa that produce them), we tested for homogeneity of group dispersions based on Bd infection status using the function betadisper in the vegan package (Anderson, 2006; Anderson et al., 2006). Betadisper analyses for OTUs (relative abundance) and metabolite profiles (presence/absence) were conducted separately, but, for each of these datasets, using the data for all three species combined. Prior

to analysis, OTU and metabolite data were converted to distance matrices based on Bray–Curtis and Jaccard dissimilarities, respectively. To visualize these results, we used NMDS.

Bd arrival time across sites

There is evidence that Bd has moved across Panamá from west to east (Lips et al., 2006). Bd was detected near our western most site in January 2007 and is thought to have arrived there in ∼2006 (Woodhams et al., 2008). It most likely arrived in our eastern most site in ∼2011 (Rebollar et al., 2014), although the precise dates are not known. We hypothesized that this gradient of arrival time should be correlated with aspects of the structure (OTUs) and function (metabolite profiles) of the skin microbiome along the gradient. To test this, we focused on the two treefrog species that were sampled at all four of our sites. For each of these species, we assessed variation in OTU richness, Faith's phylogenetic diversity, and metabolite richness relative to site using linear models. We assessed variation in Simpson Index using beta regression models (package betareg; Cribari-Neto and Zeileis, 2010). If analyses indicated significant differences among sites, we conducted *post-hoc* comparisons, as outlined above for species comparisons, to determine which sites were similar or different from one another. We also assessed changes in OTU community structure and metabolite profiles across this gradient using PERMANOVA. Finally, to test whether the presence of Bd decreases variation in structure and function over time, we assessed homogeneity of group dispersions (function betadisper) in OTU community structure and metabolite profiles along the gradient.

Structure-Function links and Bd arrival time

We hypothesized that longer-term presence of Bd at a site would drive a stronger correlation between structure and function of the amphibian skin microbiota as selection for strong anti-fungal producing bacterial species occurs over time. Therefore, we expected to see a tighter correlation between OTU community structure and metabolite profiles at our western sites. To assess this, we used Mantel tests to determine if there were correlations between OTU diversity and metabolite diversity for each treefrog species. OTU and metabolite data were converted to Bray–Curtis and Jaccard dissimilarities, respectively. We then compared the strength of the correlation (i.e., the Mantel statistic) across the four sites along our west-east gradient.

RESULTS

OTU and Metabolite Diversity on Amphibians from Panamá

We identified 3138 OTUs from *A. callidryas* ($N = 62$ frogs), 2704 OTUs from *D. ebraccatus* ($N = 53$ frogs), and 2667 OTUs from *C. fitzingeri* ($N = 21$ frogs), based on our 16S rRNA amplicon sequencing. Prominent phyla included Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Cyanobacteria, Verrucomicrobia, and Acidobacteria (**Figure 3A**).

Alpha diversity differed among the three species, with *C. fitzingeri* harboring the most diverse communities of bacteria in terms of richness (Chisq $= 57.3$, $P < 0.001$; *post-hoc* comparisons, *A. callidryas–D. ebraccatus*, $P = 0.002$;

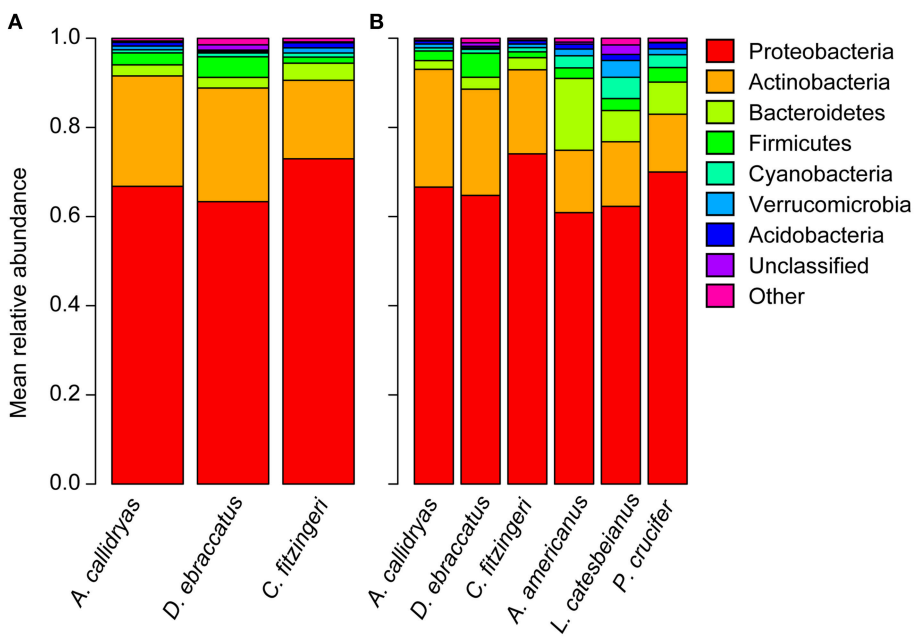

FIGURE 3 | Mean relative abundance of bacterial phyla present on the skin of (A) three frog species surveyed in Panamá in 2012 (*Agalychnis callidryas, Dendropsophus ebraccatus, Craugastor fitzingeri*; this study) and (B) a subset of individuals of each of the three frog species surveyed in Panamá compared to three frog species surveyed in Virginia, USA (*Anaxyrus americanus, Lithobates catesbeianus, Pseudacris crucifer*; Walke et al., 2015; Belden et al. unpublished data).

A. callidryas–C. fitzingeri, $P < 0.001$; *D. ebraccatus–C. fitzingeri,* $P < 0.001$; unique OTUs/individual, adjusted mean ± sd: *A. callidryas,* 364 ± 129; *D. ebraccatus*, 290 ± 57; *C. fitzingeri*, 594 ± 202) and phylogenetic diversity (Chisq = 32.4, $P < 0.001$; *post-hoc* comparisons, *A. callidryas–D. ebraccatus,* $P = 0.04$; *A. callidryas–C. fitzingeri,* $P < 0.001$; *D. ebraccatus–C. fitzingeri,* $P < 0.001$; phylogenetic diversity, adjusted mean ± sd: *A. callidryas,* 15.9 ± 5.1; *D. ebraccatus*, 13.7 ± 3.1; *C. fitzingeri*, 22.6 ± 5.3). Although diverse, the bacterial communities of all three species were typically uneven and dominated by a small number of OTUs (Simpson, adjusted mean ± sd: *A. callidryas,* 0.87 ± 0.07; *D. ebraccatus*, 0.88 ± 0.05; *C. fitzingeri*, 0.81 ± 0.08). However, relative to *A. callidryas* and *D. ebraccatus*, the communities of *C. fitzingeri* tended to be more even (as evaluated by Simpson Index; Chisq = 7.9, $P = 0.02$; *post-hoc* comparisons, *A. callidryas–D. ebraccatus,* $P = 0.86$; *A. callidryas–C. fitzingeri,* $P = 0.06$; *D. ebraccatus–C. fitzingeri,* $P = 0.03$).

On average, the top three OTUs in terms of relative abundance accounted for about 50% of the total relative abundance on a given frog (range 9–70%). A comparison of the top three OTUs present on every individual of each species revealed one OTU that dominated the communities of all three species (X394796, Proteobacteria: Pseudomonadaceae; dominant on 88% of individuals; relative abundance/species: mean ± sd: *A. callidryas,* 15 ± 10%, *D. ebraccatus,* 12 ± 9%, *C. fitzingeri,* 32 ± 12%). Two additional OTUs were dominant in the communities of *A. callidryas* and *D. ebraccatus* (X4451011, Proteobacteria: Pseudomonadaceae dominant on 85 and 79% of individuals of *A. callidryas* and *D. ebraccatus*, respectively; relative abundance/species: mean ± sd: *A. callidryas,* 12 ± 7%, *D. ebraccatus,* 13 ± 8%; X235695, Actinobacteria: Cellulomonadaceae dominant on 42 and 57% of individuals of *A. callidryas* and *D. ebraccatus*, respectively; relative abundance/species: mean ± sd: *A. callidryas* 17 ± 19%, *D. ebraccatus* 20 ± 18%). By contrast, *C. fitzingeri* harbored a high abundance of two different OTUs (X4473756, Actinobacteria: Cellulomonadaceae dominant on 76% of individuals; relative abundance, mean ± sd: 7 ± 3% and X1139932, Proteobacteria: Xanthomonadaceae dominant on 67% of individuals; relative abundance, mean ± sd: 6 ± 2%).

Based on NMDS ordination, there were differences in OTU community structure among the three frog species when accounting for site (**Figure 4A**, NMDS stress: 0.11, Adonis pseudo = 12.92, $R^2 = 0.16$, $P = 0.001$; *post-hoc* comparisons,

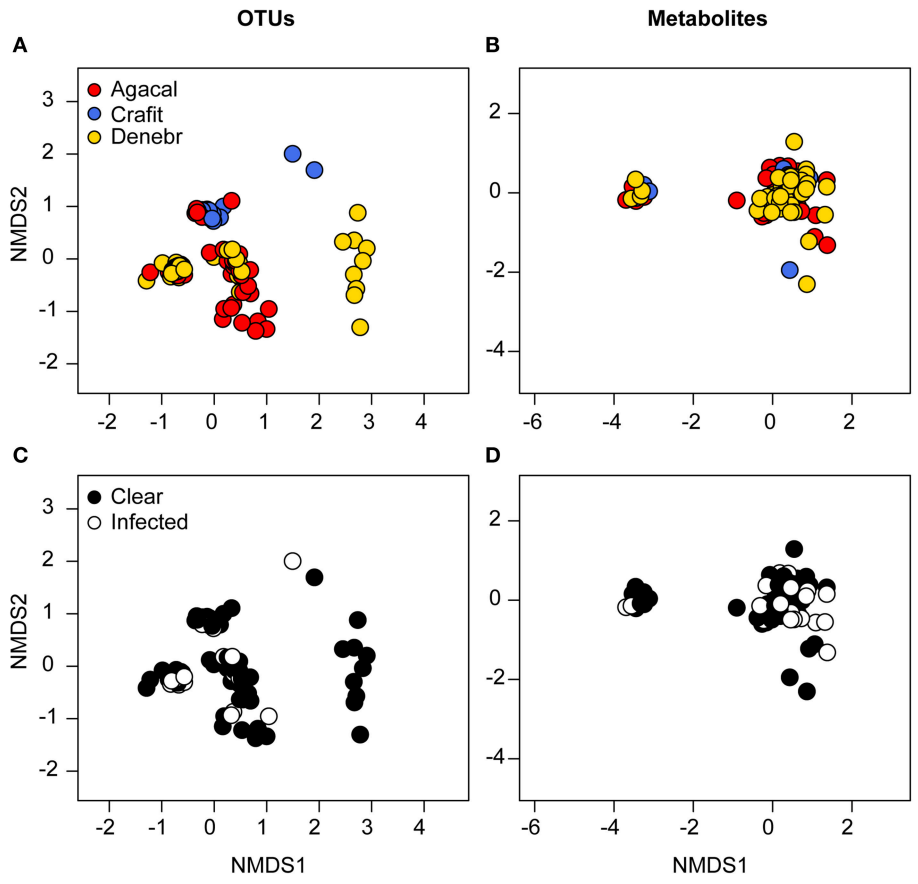

FIGURE 4 | Beta diversity of bacterial communities (OTUs) (left column) and bacterially-produced metabolite profiles (right column) sampled from the skin of three frog species from Panamá, grouped by frog species (A,B, red, *Agalychnis callidryas*; gold, *Dendropsophus ebraccatus*; blue, *Craugastor fitzingeri*) and the frog's infection status (C,D, white, infected; black, not infected) by the fungal pathogen, *Batrachochytrium dendrobatidis*. NMDS ordinations are based on Bray–Curtis and Jaccard dissimilarities for OTUs and metabolites, respectively. Each point represents a single individual.

A. callidryas–D. ebraccatus Adonis pseudo = 2.43, $R^2 = 0.02$, $P = 0.008$; *A. callidryas–C. fitzingeri* Adonis pseudoF = 20.4, $R^2 = 0.20$, $P = 0.001$; *D. ebraccatus–C. fitzingeri* Adonis pseudoF = 23.2, $R^2 = 0.24$, $P = 0.001$). K-S measures of the final set of 41 species-defining OTUs ranged from 0.40 to 0.60 (**Table 2**). Samples of *C. fitzingeri* were clearly distinguishable based on these OTUs; many K-S-defined OTUs that commonly occurred in high abundance in samples of *C. fitzingeri* were rare on the two treefrogs, and vice versa (i.e., OTUs that were rare on *C. fitzingeri* were abundant on the treefrogs; **Figure 5**). Differences between the treefrogs were more subtle. Exceptions include an OTU in the family Alcaligenaceae (X820379), found almost exclusively on *A. callidryas,* and an unclassified OTU in the candidate phylum GN02 (denovo129256) that was abundant in samples of *D. ebraccatus* (**Figure 5**).

We identified a total of 163 unique metabolites from the skin of our three frog species from Panamá (total number of metabolites/species: *A. callidryas*, 135; *D. ebraccatus*, 120; *C.*

TABLE 2 | K-S Measures and taxonomic information for 41 OTUs that best defined the differences in skin bacterial community structure of three frog species from Panamá: *Agalychnis callidryas*, *Dendropsophus ebraccatus*, and *Craugastor fitzingeri*.

OTU	K-S measure	Phylum	Class	Order	Family	Genus
X4473756	0.60	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Unclassified
X926370	0.59	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
X845178	0.59	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X71872	0.59	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Comamonas*
X268968	0.59	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*
X4378239	0.58	Actinobacteria	Actinobacteria	Actinomycetales	Sanguibacteraceae	*Sanguibacter*
X4451011	0.58	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X563957	0.58	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Unclassified
X107523	0.58	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X4469492	0.55	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X4453998	0.55	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X429048	0.55	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Unclassified
X1139932	0.55	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Unclassified
X4449458	0.55	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
X3979725	0.54	Actinobacteria	Actinobacteria	Actinomycetales	Unclassified	Unclassified
X7821	0.52	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas acidaminiphila*
X817507	0.52	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Unclassified
X394796	0.52	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
X820379	0.51	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Unclassified
X1109251	0.50	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
X410048	0.49	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
X3167757	0.48	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X2685602	0.47	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X2360704	0.46	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X4432930	0.45	Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075	Unclassified
X4422388	0.45	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X748412	0.44	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*
X4370747	0.44	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
denovo50614	0.43	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
denovo5853	0.43	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Unclassified
X2458172	0.43	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Ochrobactrum*
X72153	0.42	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Unclassified
denovo64223	0.42	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Unclassified
X1119668	0.42	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Devosia*
X254649	0.42	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Unclassified
X269930	0.42	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas veronii*
denovo129256	0.42	GN02	BD1-5	Unclassified	Unclassified	Unclassified
X4373617	0.41	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Unclassified
X4452118	0.41	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X4333206	0.40	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*
X813954	0.40	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Devosia*

The K-S Measure ranges from 0 to 1, where values closer to one imply a greater difference between the K distributions than values closer to zero. OTUs are listed in order of descending K-S Measure. Most OTUs were unclassified at the species level; if species information was available, it is listed along with the genus in the Genus column.

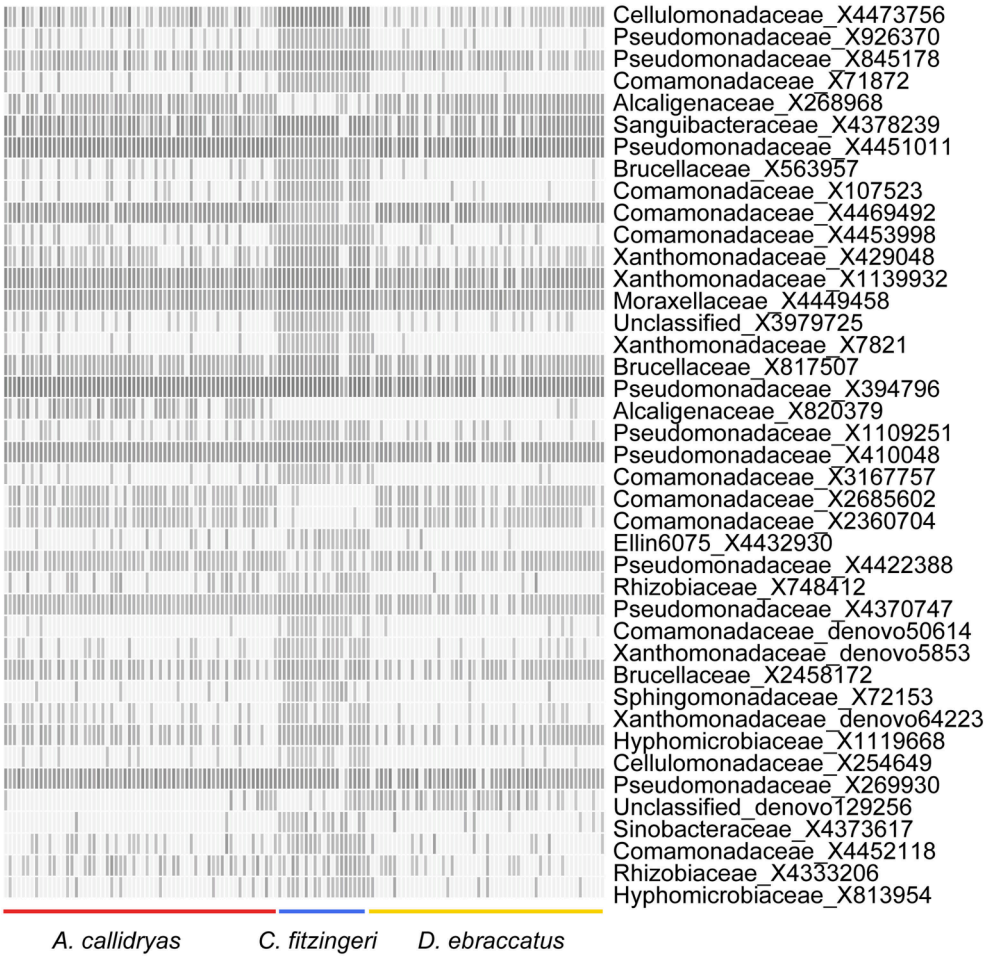

FIGURE 5 | Relative abundance of 41 OTUs selected based on K-S measures that best defined the differences in skin bacterial community structure of three frog species from Panamá (left, *Agalychnis callidryas*; right, *Dendropsophus ebraccatus*; center, *Craugastor fitzingeri*). OTU relative abundances ranged from 0 to 0.32. Lighter shades indicate lower relative abundances (white relative abundance = 0) and darker shades indicate higher relative abundances (darkest relative abundance = 0.32). OTUs are ordered top to bottom based on K-S measures (see **Table 2** for exact values and additional taxonomic information for each OTU).

fitzingeri, 105) based on the HPLC-MS analysis. Metabolite richness differed across the three species (Chisq = 6.3, $P = 0.04$). Richness values were on average highest for *A. callidryas* and lowest for *D. ebraccatus* (*post-hoc* comparisons: *A. callidryas*–*C. fitzingeri*, $P = 0.6$; *A. callidryas*–*D. ebraccatus*, $P = 0.03$; *D. ebraccatus*–*C. fitzingeri*, $P = 0.6$; richness, adjusted mean ± sd: *A. callidryas*, 32 ± 7; *D. ebraccatus*, 28 ± 8; *C. fitzingeri*, 30 ± 8); however, the number of metabolites associated with any given individual was variable for all three species (range: 13–47 metabolites for *A. callidryas*, 8–50 for *D. ebraccatus*, and 16–52 for *C. fitzingeri*). Metabolite profiles also differed among the three species when accounting for site (**Figure 4B**, NMDS stress: 0.12, Adonis pseudoF = 1.9, $R^2 = 0.03$, $P = 0.02$; *post-hoc* comparisons, *A. callidryas*–*D. ebraccatus* Adonis pseudoF = 2.2, $R^2 = 0.02$, $P = 0.01$; *A. callidryas*–*C. fitzingeri* Adonis pseudoF = 2.3, $R^2 = 0.03$, $P = 0.05$; *D. ebraccatus*–*C. fitzingeri* Adonis pseudoF = 1.16, $R^2 = 0.02$, $P = 0.35$), although the separation was not as clear on the NMDS as it was for the OTUs (PERMANOVA: $R^2 = 0.03$ for metabolites vs. $R^2 = 0.16$ for OTUs).

Comparison of the Diversity of Skin Microbial Communities of Amphibians from Panamá and the US

Despite wide geographic separation and likely variation in the history of Bd presence, at the phylum level, similar taxa dominated the skin bacterial communities of amphibians from Panamá and the US. The Proteobacteria accounted for over 60% of the relative abundance of the bacterial assemblages for all species (**Figure 3B**; relative abundance, mean ± sd: *A. callidryas* 67 ± 15%, *D. ebraccatus* 65 ± 15%, *C. fitzingeri* 74 ± 5%, *A. americanus* 61 ± 9%, *L. catesbeianus* 62 ± 13%, *P. crucifer* 70 ± 15%). The Actinobacteria accounted

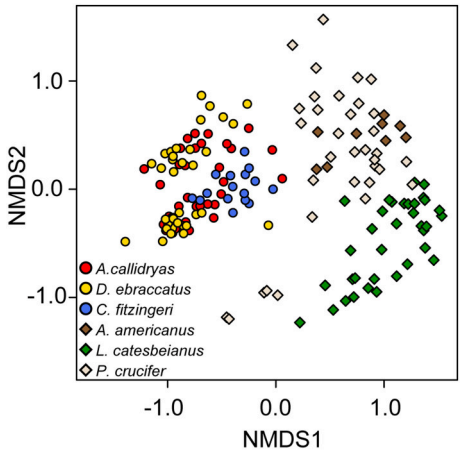

FIGURE 6 | Beta diversity of skin bacterial communities of tropical (circles) and temperate (diamonds) amphibians. Three species from each zone were included in the analysis [red, *Agalychnis callidryas*; gold, *Dendropsophus ebraccatus*; blue, *Craugastor fitzingeri* from Panamá (tropical zone); brown, *Anaxyrus americanus*; green, *Lithobates catesbeianus*; cream, *Pseudacris crucifer* from Virginia, USA (temperate zone)]. NMDS ordination based on Bray–Curtis dissimilarities. Each point represents a single individual.

for the second largest proportion of the relative abundance of all three species from Panamá, whereas the Actinobacteria and Bacteroidetes were more evenly represented in terms of relative abundance for the three species from the US (**Figure 3B**).

In terms of richness and phylogenetic diversity, the bacterial communities on the skin of the temperate and tropical amphibians we examined were similar (richness, Chisq = 2.9, $P = 0.09$; unique OTUs/individual, adjusted mean ± sd: Temperate species 609 ± 279; Tropical species, 401 ± 165; phylogenetic diversity, Chisq = 0.216, $P = 0.64$, adjusted mean ± sd: Temperate species 19.7 ± 7.8; Tropical species, 16.3 ± 4.4). Overall, the communities of tropical species were slightly more even than those of temperate species (Simpson Index, Chisq = 4.3, $P = 0.04$, adjusted mean ± sd: Temperate species 0.88 ± 0.09; Tropical species, 0.86 ± 0.05).

While 68% (3228/4698) of the OTUs in this dataset were found on frogs in both the temperate and tropical zones, the skin bacterial communities of temperate and tropical amphibians were distinct (**Figure 6**, NMDS stress: 0.15, Adonis pseudoF = 49.73, $R^2 = 0.23$, $P = 0.001$). Tropical and temperate frog species clustered separately on the NMDS plot, but they also varied in dispersion, with temperate frogs seeming to have much more variance in the skin bacterial community as compared to the tropical frogs (test of beta-dispersion, $F = 84.56$, $P = 0.001$). Given these differences, we used the K-S measure to identify some of key OTUs that contributed the most to this difference among the temperate and tropical frogs we sampled. K-S measures for the final set of 31 OTUs defining tropical and temperate species ranged from 0.38 to 0.50 (**Table 3**). Most of the K-S defined OTUs were present in both zones, but exhibited higher relative abundance distributions for the tropical species (**Figure 7**). However, several OTUs exhibited complete separation by zone (**Figure 7**). Specifically, two OTUs in the family Pseudomonadaceae, X4370747 and X272189, were unique to tropical species, whereas four OTUs in the family Pseudoalteromonadaceae—X4406967, X1105883, X309489, and X4353625—were only found on temperate species (**Figure 7**).

Impact of Bd on Amphibian Microbiome Structure and Function: Hypothesis Tests

Bd Infection Status

A total of 35 frogs were infected with Bd in our survey, with 3–4 individuals infected/species/site on average (details in Table 2 in Rebollar et al., 2014). Treefrog *Bd* infection intensities (zoospore equivalents) across the four sites varied from 0.09 to 0.51 for the 16 infected *A. callidryas* and 0.15–0.24 for the 12 infected *D. ebraccatus* (reported in Table 2 in Rebollar et al., 2014). In addition, Bd infection intensities for the seven infected *C. fitzingeri* across two sites varied from 0.05 to 36.56 (reported in Table 2 in Rebollar et al., 2014). Bd infection status of individuals was not associated with changes in alpha diversity of either OTUs or metabolite profiles (OTU richness: Chisq = 0.02, $P = 0.9$; phylogenetic diversity: Chisq = 0.01, $P = 0.9$; metabolite richness: Chisq = 1.2, $P = 0.3$). Bd-infected individuals also did not cluster separately from uninfected individuals based on NMDS analyses of the structure (OTUs) and function (metabolite profiles) of the skin microbial communities after accounting for "species × site" level effects (OTUs, **Figure 4C**, NMDS stress: 0.11, Adonis pseudoF = 2.6, $R^2 = 0.05$, $P = 0.23$; metabolites, **Figure 4D**, NMDS stress: 0.12, Adonis pseudoF = 0.81, $R^2 = 0.01$, $P = 0.76$). Some caution should be used when inferring from these results relating to Bd infection status due to low statistical power when accounting for species and site level effects in the models. For example, approximate power calculations for the Bd effect size in richness when accounting for site and species are on the order of 10–20%. Thus, if we were to sample frogs with the same rate of infection (∼1 Bd-infected frog among 5 frogs), we would need to sample >350 frogs to improve our power to 80%.

Bd Arrival Time Across Sites

Alpha-diversity of skin bacterial communities, in terms of OTU richness, phylogenetic diversity, and evenness varied significantly across the four sites for both species of treefrogs (**Figures 8A–F**, *A. callidryas*, OTU richness: $F = 6.75$, $P < 0.001$, phylogenetic diversity: $F = 3.55$, $P = 0.02$, Simpson index: Chisq = 53.3, $P < 0.001$; *D. ebraccatus*, OTU richness: $F = 4.46$, $P = 0.007$, phylogenetic diversity: $F = 6.01$, $P = 0.001$, Simpson index: Chisq = 69.9, $P < 0.001$). Metabolite richness did not vary across sites (**Figures 8G,H**, *A. callidryas*, $F = 1.11$, $P = 0.35$; *D. ebraccatus*, $F = 1.33$, $P = 0.27$). However, while there was variation in OTU alpha-diversity across sites, the variation was not consistent with changes that would have been expected with the movement of Bd from western to eastern sites. For *A. callidryas*, OTU richness and phylogenetic diversity was highest at the eastern most site (most recent Bd arrival, Nuevo Vigia; *post-hoc* tests summarized in **Table 4**). However,

TABLE 3 | K-S Measures and taxonomic information for 31 OTUs that best defined the differences in skin bacterial community structure of tropical and temperate amphibians.

OUT	K-S measure	Phylum	Class	Order	Family	Genus
X4406967	0.50	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	*Pseudoalteromonas*
X410048	0.48	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X394796	0.48	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
X81358	0.48	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Rhodanobacter*
X4449458	0.48	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
X4353625	0.47	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	*Pseudoalteromonas*
X4451011	0.47	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X4370747	0.47	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
X269930	0.47	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas veronii*
X2119418	0.46	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Unclassified
X309489	0.46	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	*Pseudoalteromonas*
X817507	0.45	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Unclassified
X272189	0.45	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
X1139932	0.45	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Unclassified
X1105883	0.44	Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	*Pseudoalteromonas*
X4349788	0.44	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas viridiflava*
X814442	0.44	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*
X4469492	0.43	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X4422388	0.42	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X4430952	0.42	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*
X4347599	0.42	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	*Vibrio*
X4388545	0.41	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Rhodoferax*
X4456891	0.41	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
X142419	0.41	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
X360440	0.40	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter rhizosphaerae*
denovo74396	0.40	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unclassified
X668514	0.39	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Unclassified
X4364813	0.39	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Unclassified
X235695	0.39	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Cellulomonas*
X219151	0.38	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
X2458172	0.38	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Ochrobactrum*

The K-S Measure ranges from 0 to 1, where values closer to one imply a greater difference between the K distributions than values closer to zero. Three species from each zone were included in the analysis: Agalychnis callidryas, Dendropsophus ebraccatus, Craugastor fitzingeri from Panamá (tropical zone) and Anaxyrus americanus, Lithobates catesbeianus, Pseudacris crucifer from Virginia, USA (temperate zone). OTUs are listed in order of descending K-S Measure. Most OTUs were unclassified at the species level; if species information was available, it is listed along with the genus in the Genus column.

this same pattern was not seen in *D. ebraccatus*, where OTU richness only differed between the two western most sites, and phylogenetic diversity only differed between the two eastern most sites (*post-hoc* tests summarized in **Table 4**). For both treefrog species, the two central sites tended to have communities dominated by few OTUs, with more even communities at the western and eastern sites (*post-hoc* tests summarized in **Table 4**).

Beta-diversity of OTUs varied across the four sites for both treefrog species, while metabolite profiles only differed across sites for *A. callidryas* (OTUs: A. callidryas, **Figure 9A**, NMDS stress: 0.12, Adonis pseudoF = 19.25, R^2 = 0.50, P = 0.001; *D. ebraccatus*, **Figure 9B**, NMDS stress: 0.06, Adonis pseudoF = 20.24, R^2 = 0.55, P = 0.001; Metabolites: *A. callidryas*, **Figure 9C**, NMDS stress: 0.12, Adonis pseudoF = 2.17, R^2 = 0.10, P = 0.001; *D. ebraccatus*, **Figure 9D**, NMDS stress: 0.11, Adonis pseudoF = 1.40, R^2 = 0.08, P = 0.07). However, as for the alpha-diversity metrics, there was no clear indication that this variation across sites was strongly associated with the west-east gradient of Bd arrival times. For both treefrog species, the individuals from the two sites farthest west and east on our gradient (Cerro Campana and Nuevo Vigia) clustered together on the NMDS plots of OTU communities and were separate from the two central sites (**Figures 9A,B**).

We also examined whether variance (dispersion) in the OTU communities or metabolite profiles varied across sites. We found that for both treefrog species, dispersion did differ among sites for the OTUs (**Figures 10A,B**, *A. callidryas* F = 20.24, P < 0.001; *D. ebraccatus* F = 118.78, P < 0.001), but not for

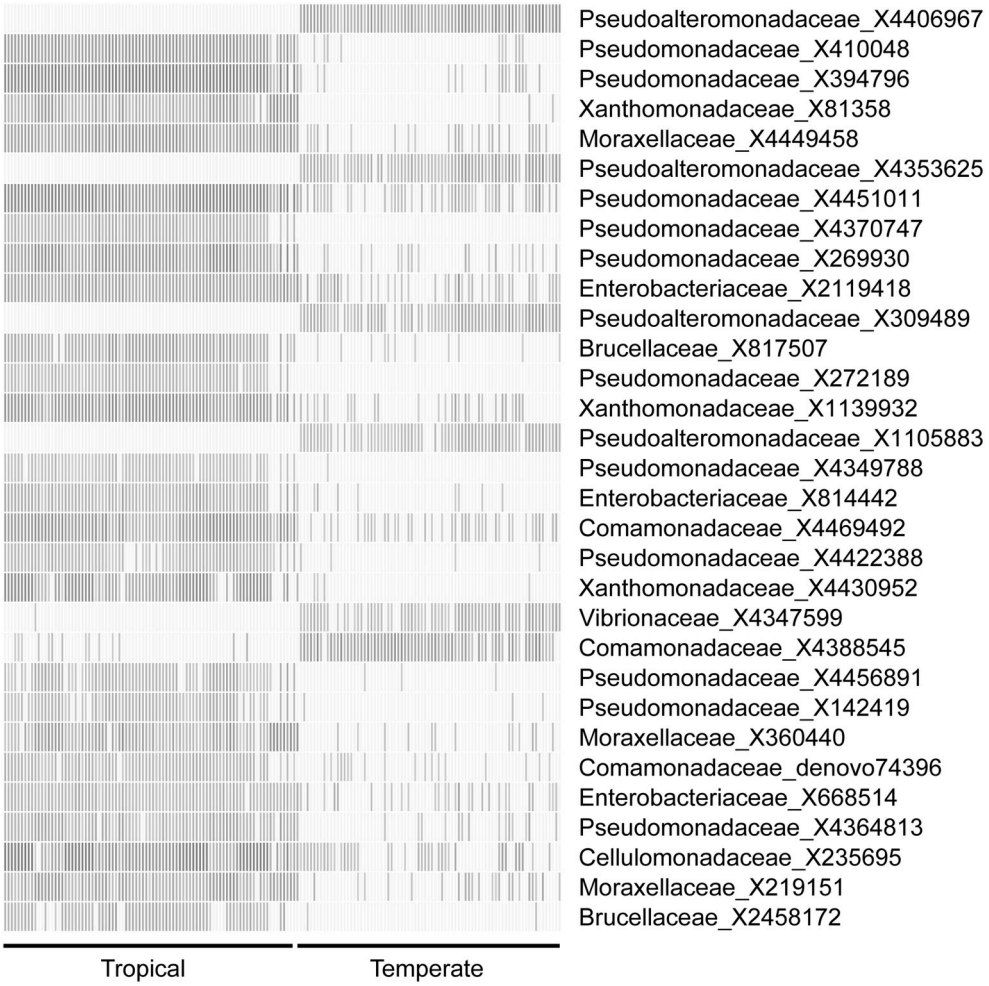

FIGURE 7 | Relative abundance of 31 OTUs selected based on K-S measures that best defined the differences in skin bacterial community structure of tropical (left) and temperate (right) amphibians. Three species from each zone were included in the analysis: *Agalychnis callidryas*, *Dendropsophus ebraccatus*, *Craugastor fitzingeri* from Panamá (tropical zone) and *Anaxyrus americanus, Lithobates catesbeianus, Pseudacris crucifer* from Virginia, USA (temperate zone). OTU relative abundances ranged from 0 to 0.37. Lighter shades indicate lower relative abundances (white, relative abundance = 0) and darker shades indicate higher relative abundances (darkest relative abundance = 0.37). OTUs are ordered top to bottom based on K-S measures (see **Table 3** for exact values and additional taxonomic information for each OTU).

the metabolites (*A. callidryas* metabolites, $F = 1.9$, $P = 0.14$; *D. ebraccatus* $F = 0.05$, $P = 0.99$). This pattern again did not seem to correlate linearly with the west-east gradient. The two central sites had higher dispersion than the two extreme sites, which seemed to match the pattern for beta-diversity based on the Adonis tests. This is not surprising, as the multivariate permutational tests can be influenced by both mean tendency and dispersion.

Structure-function Links and Bd Arrival Time

There was no correlation between structure (OTUs) and function (metabolites) for either of the treefrog species at any of the four sites (**Table 5**); therefore we did not pursue examining changes in the relationship from west to east.

DISCUSSION

We found clear differences in the skin microbiome of the three tropical frog hosts we assessed, including for the two treefrog species that were sampled from the same ponds at the four different sites. The most pronounced differences in relative abundance distributions occurred between the robber frog (*C. fitzingeri*) and the two treefrogs (*A. callidryas* and *D. ebraccatus*). The two treefrogs tended to have more similar relative abundance distributions, suggesting that phylogenetic relatedness and/or habitat overlap may explain some of the variation in skin bacterial community structure. These species-level differences in the amphibian skin microbiota have been documented previously, although primarily from temperate amphibian species (in Colorado, US, McKenzie et al., 2012; in California, US, Kueneman et al., 2014; in Virginia, US,

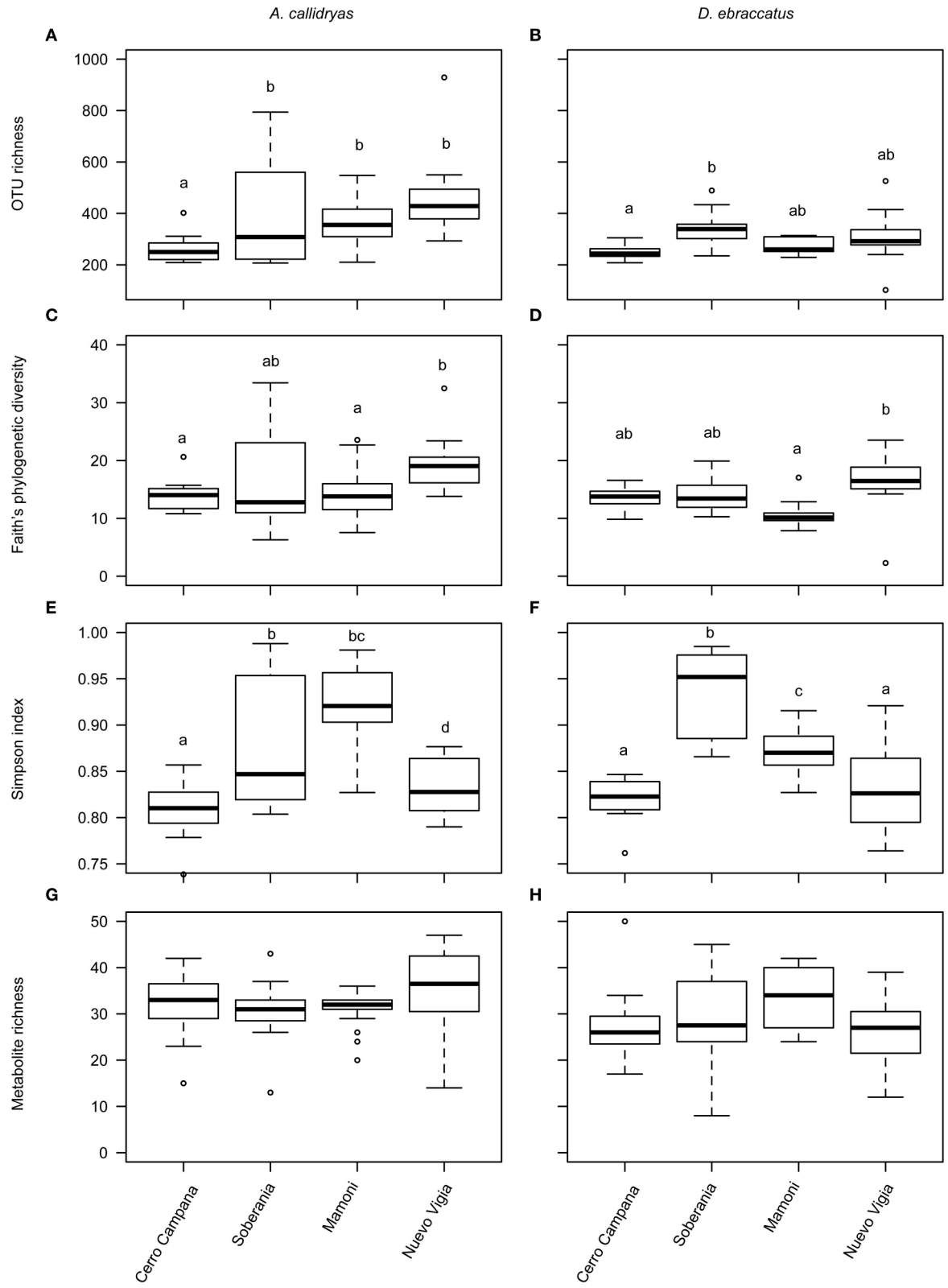

FIGURE 8 | Alpha diversity of bacterial OTUs (A,B, richness; C,D, phylogenetic diversity; E,F, evenness) and bacterially-produced metabolites (G,H, richness) sampled from the skin of *Agalychnis callidryas* (left column) and *Dendropsophus ebraccatus* (right column). Both species were sampled at each of four locations during a field survey conducted in Panamá in 2012. Sites are ordered left to right along the x-axis based on the relative length of time these species have persisted with the fungal pathogen, *Batrachochytrium dendrobatidis*, at each site (i.e., longest at Cerro Campana and shortest at Nuevo Vigia; Woodhams et al., 2008; Rebollar et al., 2014). The letters above the bars indicate statistically significant differences among sites.

TABLE 4 | Results of *post-hoc* comparisons indicating if OTU alpha diversity measures (richness, phylogenetic diversity, Simpson Index) were similar or different among four sites sampled during a field survey assessing the diversity of bacterial communities on amphibian skin.

	Estimate	Std. Error	Test statistic	*P*-value
***A. callidryas* RICHNESS**				
Soberania—CerroCampana	0.33	0.119	2.778	**0.036**
Mamoni—CerroCampana	0.336	0.111	3.026	**0.019**
NuevoVigia—CerroCampana	0.551	0.126	4.372	**<0.001**
Mamoni—Soberania	0.006	0.111	0.056	0.999
NuevoVigia—Soberania	0.221	0.126	1.753	0.3055
NuevoVigia—Mamoni	0.215	0.118	1.807	0.28
***D. ebraccatus* RICHNESS**				
Soberania—CerroCampana	0.293	0.082	3.554	**0.005**
Mamoni—CerroCampana	0.079	0.944	0.845	0.832
NuevoVigia—CerroCampana	0.156	0.081	1.929	0.229
Mamoni—Soberania	-0.214	0.948	-2.258	0.122
NuevoVigia—Soberania	-0.137	0.082	-1.659	0.355
NuevoVigia—Mamoni	0.077	0.093	0.826	0.841
***A. callidryas* PHYLOGENETIC DIVERSITY**				
Soberania—CerroCampana	2.569	1.853	1.386	0.512
Mamoni—CerroCampana	0.267	1.734	0.169	0.998
NuevoVigia—CerroCampana	5.583	1.966	2.841	**0.031**
Mamoni—Soberania	-2.276	1.734	-1.313	0.558
NuevoVigia—Soberania	3.015	1.966	1.534	0.424
NuevoVigia—Mamoni	5.291	1.853	2.855	0.294
***D. ebraccatus* PHYLOGENETIC DIVERSITY**				
Soberania—CerroCampana	0.464	1.217	0.382	0.981
Mamoni—CerroCampana	-2.763	1.381	-2.001	0.201
NuevoVigia—CerroCampana	3.002	1.196	2.511	0.07
Mamoni—Soberania	-3.227	1.399	-2.307	0.11
NuevoVigia—Soberania	2.538	1.217	2.085	0.717
NuevoVigia—Mamoni	5.765	1.381	4.175	**<0.001**
***A. callidryas* SIMPSON INDEX**				
Soberania—CerroCampana			13.4	**<0.001**
Mamoni—CerroCampana			38.7	**<0.001**
NuevoVigia—CerroCampana			4.7	**0.03**
Mamoni—Soberania			1.0	0.3
NuevoVigia—Soberania			7.0	**0.008**
NuevoVigia—Mamoni			24.9	**<0.001**
***D. ebraccatus* SIMPSON INDEX**				
Soberania—CerroCampana			33.2	**<0.001**
Mamoni—CerroCampana			18.2	**<0.001**
NuevoVigia—CerroCampana			1.6	0.2
Mamoni—Soberania			11.9	**<0.001**
NuevoVigia—Soberania			23.3	**<0.001**
NuevoVigia—Mamoni			3.7	**0.05**

Results are presented for the two species, Agalychnis callidryas and Dendropsophus ebraccatus, that were sampled at all four sites. For richness and phylogenetic diversity the reported test statistic is t, while for Simpson Index the reported test statistic is Chi-Square. Bolded P-values are statistically significant.

Walke et al., 2014). In tropical systems, differences in the skin microbiota among three *Atelopus* spp. frogs have been seen in Colombia using culture-based methods (Flechas et al., 2012).

These host species differences in the amphibian microbiota are one of the clearest patterns to have emerged from the studies completed to date. The mechanisms driving host species differences are not well understood, although it is likely a combination of differences in environmental reservoirs of bacteria, as well as host and microbe factors that contribute to community assembly of the skin microbiota. In the salamander *Plethodon cinereus*, the structure of the environmental bacterial community appears to be a major determinant of bacterial community structure of the salamanders' skins (Loudon et al., 2014). However, while various members of the amphibian skin microbiota may be found in the environment, environmental differences in the source pool of exposure do not completely explain differences among amphibian host species, as even species inhabiting the same habitats, such as ponds, can have markedly different skin bacterial communities (McKenzie et al., 2012; Walke et al., 2014). Although there has not been extensive work done on species-level differences in the microbiome among other free-living animals, similar species differences have been observed in a few systems, including in marine sponges (Schmitt et al., 2012; Easson and Thacker, 2014), *Hydra* (Franzenburg et al., 2013), and primates (Yildirim et al., 2010).

Only a very small fraction of OTUs were dominant on frog skins despite a large number of total OTUs observed. With this pattern in mind, we can hypothesize that only a small number of OTUs are involved with the host in a stable mutualism, while other OTUs are individually rare and more transient. However, the sum total of the more rare community likely competes with the relatively abundant OTUs, thereby altering the competitive landscape and preventing one OTU from achieving competitive exclusion (Loudon et al., 2014). A focus on the biology of the function of the dominant OTUs is likely to advance our knowledge of how maintaining these symbiotic bacteria might benefit the host.

We also observed host species differences in metabolite diversity, with *A. callidryas* having the most metabolites of the three species we examined. There was also an indication of species differences in the metabolite profiles, but the pattern of separation of the three species was not as clear as it was for the OTU data. The lack of clear species differentiation in metabolite profiles was surprising because in a prior study using the same HPLC-MS methods, Umile et al. (2014) saw very clear differences in the metabolite profiles across 10 amphibian species. Two key distinctions between that study and the present study were that (1) we rinsed frogs to remove dirt and transient microbes prior to collecting the swab samples, which was not done in Umile et al. (2014) and (2) we collected two swab samples, with the second swab always dedicated to the metabolite analyses and the first to the bacterial OTU analysis, while Umile et al. (2014) alternated swab order for bacteria and metabolites. So while we still did see a signal of species level differences in our study, we think that one or both of these factors might have masked that signal relative to the findings of Umile et al. (2014).

In addition to the species level differences in OTU structure within Panamá, we also saw clear separation between the tropical and temperate species in our analysis when comparing the OTUs on the Panamanian frogs with frogs collected in Virginia, US. Indeed, the differences in the relative abundance distributions

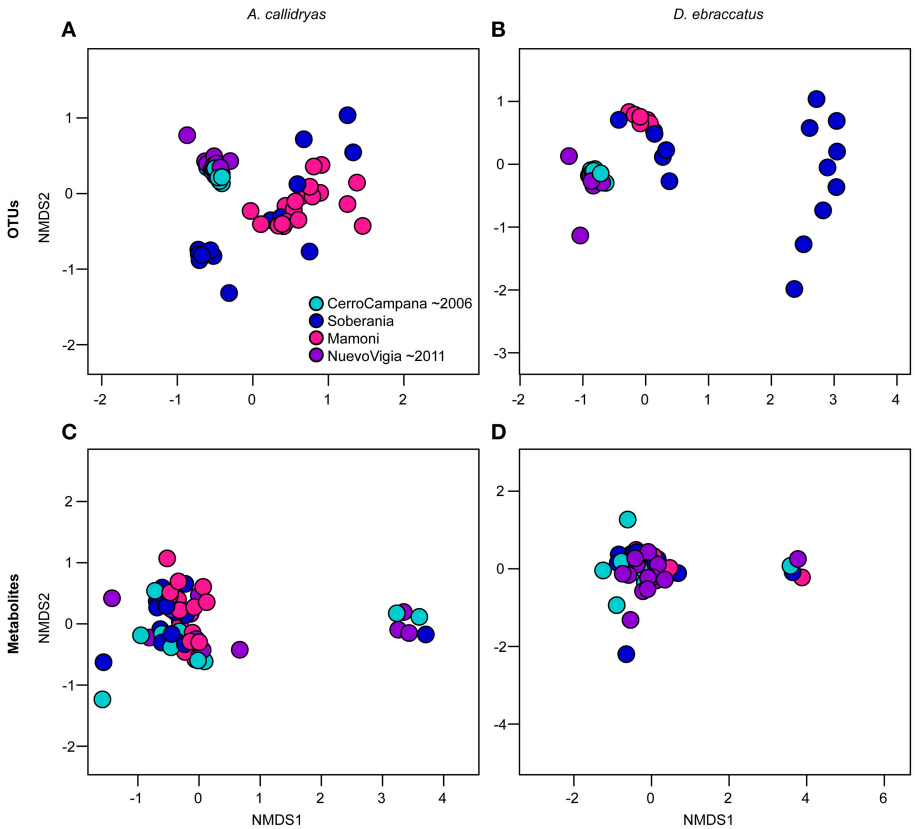

FIGURE 9 | Beta diversity of bacterial communities (OTUs) (A,B) and bacterially-produced metabolite profiles (C,D) sampled from the skin *Agalychnis callidryas* (left column), *Dendropsophus ebraccatus* (right column), grouped by sampling location. Both species were sampled at each of four locations during a field survey conducted in Panamá in 2012. The color of the circles indicates the four sites and relative length of time these species have persisted with the fungal pathogen, *Batrachochytrium dendrobatidis*, at each site (i.e., aquamarine = longest at Cerro Campana and purple = shortest at Nuevo Vigia; Woodhams et al., 2008; Rebollar et al., 2014). NMDS ordinations are based on Bray–Curtis and Jaccard dissimilarities for OTUs and metabolites, respectively. Each point represents a single individual.

were obvious for most of the 31 OTUs identified by our K-S approach. At the level of phyla, however, there was broad similarity between Panamá and US frog skin bacteria, with dominance of Proteobacteria and Actinobacteria, and lesser contribution of OTUs in the Bacteroidetes and Firmicutes, although the relative abundances of these groups seemed to shift somewhat across zones. We made no attempt to control for phylogeny in our analysis although we did have Hylids in both groups with *A. callidryas* and *D. ebraccatus* from Panamá and *P. crucifer* from the US, and a future, larger study should do that. Few studies in other systems have investigated temperate vs. tropical host-associated microbes of related host taxa; however, in marine sponges there are distinctions between the microbiota of tropical and sub-tropical species (Schmitt et al., 2012). Our initial finding of differences among tropical and temperate species suggests that a larger study of the biogeography of amphibian skin microbes will be fruitful and might lead to new insights about interactions between Bd and the skin microbiome.

We did not find clear evidence of links between Bd and the amphibian skin microbiota. In terms of individual frogs, for instance, there were no differences in microbiota structure (OTUs) or function (metabolite profiles) based on whether frogs were Bd-positive or Bd-negative at the time of sampling. We did not have a lot of infected frogs in our survey (only 35/136 frogs), and most of these Bd infected frogs had low infection intensities. In particular, with relatively few infected frogs, and those split across three species and four sites, we had low statistical power to detect any possible differences that were based on Bd infection status. In addition, with field sampling we do not know what the individual history of each frog was in relation to Bd. Our samples likely represented a mix of frogs with active infection, some frogs with prior exposure that subsequently cleared infection, and some with no prior exposure, even within Bd endemic sites. In addition, if Bd had already selected for hosts that maintain defensive microbial communities, then we would not expect to see differences in community structure between currently infected and uninfected individuals. Experimental Bd exposure studies have demonstrated that the initial amphibian skin microbiota can influence disease outcome following Bd exposure and that Bd itself can impact the skin bacterial community structure (Harris et al., 2009; Jani and Briggs, 2014; Becker et al., 2015a). We also intentionally focused on three hosts

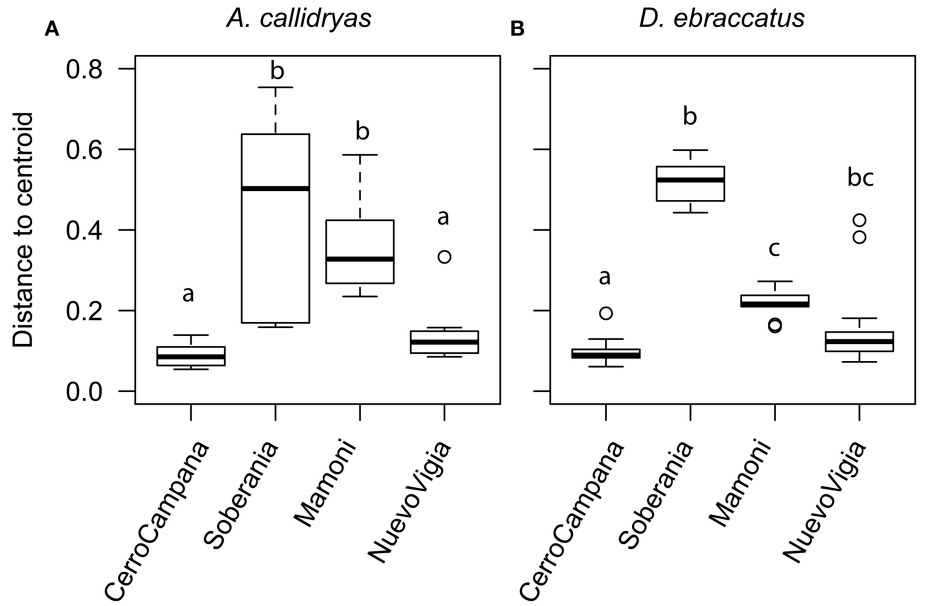

FIGURE 10 | Dispersion of the bacterial communities (OTUs) sampled from the skin of (A) *Agalychnis callidryas* and (B) *Dendropsophus ebraccatus*. Both species were sampled at each of four locations during a field survey conducted in Panamá in 2012. Sites are ordered left to right along the x-axis based on the relative length of time these species have persisted with the fungal pathogen, *Batrachochytrium dendrobatidis*, at each site (i.e., longest at Cerro Campana and shortest at Nuevo Vigia; Woodhams et al., 2008; Rebollar et al., 2014). Analyses are based on Bray–Curtis and Jaccard dissimilarities for OTUs and metabolites, respectively. The letters above the bars indicate statistically significant differences among sites.

that have not experienced major declines following the arrival of Bd so that we could focus on interactions with the microbiota. The interactions on host species that are more susceptible to Bd infection might be very different than those we observed.

We also saw no clear signal in our data associated with the estimated arrival time of Bd at the various sites across our survey. A lack of long-term data, especially at the eastern-most sites, makes definitive dating of Bd arrival times more difficult in that region. Despite that, we think our hypotheses presented a reasonable expectation of what would have been expected if the wave-like pattern of spread did happen, and we did not find strong support for any of our hypotheses based on that idea. For *A. callidryas*, there did appear to be some increase in mean OTU richness along the west-east gradient, but this was not true for the other treefrog, *D. ebraccatus*. There were also differences in OTU community structure and dispersion across the four sites for the two treefrogs, but the differences were not consistent with a pattern of change occurring from west to east. It is possible that selection on bacterial communities or amphibian hosts is strongest when Bd first arrives and occurs quite rapidly. In that case, we would not expect to see large differences as a function of arrival time of Bd, even though the eastern-most site we sampled likely had Bd present for less than a year when we sampled there. In addition to Bd arrival time, many other factors also vary among the four sites we surveyed, including precipitation patterns, elevation, and even the timing of our sampling within the course of the field season. Any or all of these factors might also impact population level differences at our field sites and warrant further investigation.

TABLE 5 | Results of Mantel tests assessing correlations between bacterial OTU diversity and bacterially-produced metabolite profiles at each of four sites sampled during a field survey assessing the diversity of the bacterial communities on amphibian skin.

	Mantel statistic (*r*)	*P*-value
Agalychnis callidryas		
Campana	0.0484	0.372
Soberania	0.7041	0.335
Mamoni	-0.08357	0.735
NuevoVigia	0.06017	0.344
Dendropsophus ebraccatus		
Campana	-0.2941	0.939
Soberania	-0.1401	0.927
Mamoni	0.05204	0.409
NuevoVigia	-0.102	0.585

Results are presented for the two treefrog species, Agalychnis callidryas and Dendropsophus ebraccatus, that were sampled at all four sites. For each species, sites are listed based on the relative length of time these species have persisted with the fungal pathogen, Batrachochytrium dendrobatidis, at each site (i.e., longest at Cerro Campana and shortest at Nuevo Vigia; Woodhams et al., 2008; Rebollar et al., 2014).

While OTU community structure varied at the different sites, we did not see clear clustering of metabolite profiles based on site, and we did not find correlations between OTU and metabolite profile distance matrices. The latter result is interesting in light of current research in ecology focused on structure-function relationships. In "macro"-systems, there is often assumed to be a positive relationship between biodiversity (often estimated

by species richness) and ecosystem function. Most tests of this idea though have focused on plant species richness, with primary production as a functional endpoint (Balvanera et al., 2006). In microbial systems, this link between structure and function may be much less pronounced due to the extreme functional redundancy that can occur in systems with thousands of species and the potential for lateral gene transfer. For example, Frossard et al. (2012) found that enzyme activity in soil bacterial communities varied little across environmental gradients despite spatial and temporal variation in bacterial community structure. Our field survey data suggest that multiple different communities of bacteria may be producing the same general sets of metabolites on frog skin, suggesting that there is not a strong link between community structure and function in this system.

AUTHOR CONTRIBUTIONS

LB, MH, KM, LH, RJ, DM, RI, and RH designed the study, MH, DM, and RI completed fieldwork in Panamá, ER, TU, EB, KM, MB, and JB processed samples in the laboratory, MH, SL, LH, and RJ completed data processing and analysis, LB and MH produced the first draft of the manuscript, and all authors edited the manuscript.

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REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austr. Ecol.* 26, 32–46. doi: 10.1046/j.1442-9993.2001.01070.x

Anderson, M. J. (2006). Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* 62, 245–253. doi: 10.1111/j.1541-0420.2005.00440.x

Anderson, M. J., Ellingsen, K. E., and McArdle, B. H. (2006). Multivariate dispersion as a measure of beta diversity. *Ecol. Lett.* 9, 683–693. doi: 10.1111/j.1461-0248.2006.00926.x

Aronesty, E. (2011). *Ea-utlls: Command-line Tools for Processing Biological Sequencing Data*. Available online at: http://code.google.com/p/ea-utils

Balvanera, P., Pfisterer, A. B., Buchmann, N., He, J. S., Nakashizuka, T., Raffaelli, D., et al. (2006). Quantifying the evidence for biodiversity effects on ecosystem functioning and services. *Ecol. Lett.* 9, 1146–1156. doi: 10.1111/j.1461-0248.2006.00963.x

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2014). *lme4: Linear Mixed-effects Models Using Eigen and S4. R Package Version 1.1-7*. Available online at: http://CRAN.R-project.org/package=lme4

Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N., and Minbiole, K. P. (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Appl. Environ. Microbiol.* 75, 6635–6638. doi: 10.1128/AEM.01294-09

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE* 5:e10957. doi: 10.1371/journal.pone.0010957

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015a). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B* 282:20142881. doi: 10.1098/rspb.2014.2881

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015b). The phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Org.* 103, 77–85. doi: 10.3354/dao02560

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Blaustein, A. R., Gervasi, S. S., Johnson, P. T., Hoverman, J. T., Belden, L. K., Bradley, P. W., et al. (2012). Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. *Philos. Trans. R. Soc. B* 367, 1688–1707. doi: 10.1098/rstb.2012.0011

Blaustein, A. R., Han, B. A., Relyea, R. A., Johnson, P. T., Buck, J. C., Gervasi, S. S., et al. (2011). The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Ann. N.Y. Acad. Sci.* 1223, 108–119. doi: 10.1111/j.1749-6632.2010.05909.x

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Boon, E., Meehan, C. J., Whidden, C., Wong, D. H. J., Langille, M. G. I., and Beiko, R. G. (2014). Interactions in the microbiome: communities of organisms and communities of genes. *FEMS Microbiol. Rev.* 38, 90–118. doi: 10.1111/1574-6976.12035

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008b). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of highthroughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Crawford, A. J., Lips, K. R., and Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in

the highlands of central Panama. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13777–13782. doi: 10.1073/pnas.0914115107

Cribari-Neto, F., and Zeileis, A. (2010). Beta regression in R. *J. Stat. Softw.* 34, 1–24. doi: 10.18637/jss.v034.i02

Da Caceres, M., Legendre, P., and Moretti, M. (2010). Improving indicator species analysis by combining groups of sites. *Oikos* 119, 1674–1684. doi: 10.1111/j.1600-0706.2010.18334.x

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Easson, C. G., and Thacker, R. W. (2014). Phylogenetic signal in the community structure of host-specific microbiomes of tropical marine sponges. *Front. Microbiol.* 5:532. doi: 10.3389/fmicb.2014.00532

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fan, J., and Lv, J. (2008). Sure independence screening for ultrahigh dimensional feature space. *J. R. Stat. Soc. B* 70, 849–911. doi: 10.1111/j.1467-9868.2008.00674.x

Fierer, N., Ferrenberg, S., Flores, G. E., Gonzalez, A., Kueneman, J., Legg, T., et al. (2012). From animalcules to an ecosystem: application of ecological concepts to the human microbiome. *Ann. Rev. Ecol. Evol. Systemat.* 43, 137–155. doi: 10.1146/annurev-ecolsys-110411-160307

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland apecies of *Atelopus*. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Fournier, D. A., Skaug, H. J., Ancheta, J., Ianelli, J., Magnusson, A., Maunder, M., et al. (2012). AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. *Optim. Methods Softw.* 27, 233–249. doi: 10.1080/10556788.2011.597854

Franzenburg, S., Walter, J., Künzel, S., Wang, J., Baines, J. F., Bosch, T. C. G., et al. (2013). Distinct antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3730–E3738. doi: 10.1073/pnas.1304960110

Frossard, A., Gerull, L., Mutz, M., and Gessner, M. O. (2012). Disconnect of microbial structure and function: enzyme activities and bacterial communities in nascent stream corridors. *ISME J.* 6, 680–691. doi: 10.1038/ismej.2011.134

Haegeman, B., Hamelin, J., Moriarty, J., Neal, P., Dushoff, J., and Weitz, J. S. (2013). Robust estimation of microbial diversity in theory and in practice. *ISME J.* 7, 1092–1101. doi: 10.1038/ismej.2013.10

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Hertz, A., Lotzkat, S., Carrizo, A., Ponce, M., Kohler, G., and Streit, B. (2012). Field notes on findings of threatened amphibian species in the central mountain range of western Panama. *Amphib. Reptile Conserv.* 6, 9–30.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometr. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jaramillo, C., Wilson, L. D., Ibáñez, R., and Jaramillo, F. (2010). "The herpetofauna of panama: distribution and conservation status," in *Conservation of Mesoamerican Amphibians and Reptiles,* eds L. D. Wilson, J. H. Townsend, and J. D. Johnson (Eagle Mountain, UT: Eagle Mountain Publishing), 604–671.

Kilburn, V. L., Ibáñez, R., Sanjur, O., Bermingham, E., Suraci, J. P., and Green, D. M. (2010). Ubiquity of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in anuran communities in Panamá. *Ecohealth* 7, 537–548. doi: 10.1007/s10393-010-0634-1

Kolmogorov, A. (1933). Sulla determinazione empirica di una legge di distribuzione. *G. Dell Inst. Ital. Delgli Attuar* 4, 83–91.

Kueneman, J. G., Parfey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Lam, B. A., Walton, D. B., and Harris, R. N. (2011). Motile zoospores of *Batrachochytrium dendrobatidis* move away from antifungal metabolites produced by amphibian skin bacteria. *Ecohealth* 8, 36–45. doi: 10.1007/s10393-011-0689-7

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Loftus, S. C., House, L. L., Hughey, M. C., Walke, J. B., Becker, M. H., and Belden, L. K. (2015). *Dimension Reduction for Multinomial Models via a Kolmogorov-Smirnov Measure (KSM)*. Blacksburg, VA: Department of Statistics, Virginia Tech. Available online at: http://www.stat.vt.edu/research/Technical_Reports/TechReport15-1.pdf

Longcore, J. E., Pessier, A. P., and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91, 219–227. doi: 10.2307/3761366

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V. J., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Manor, O., Levy, R., and Borenstein, E. (2014). Mapping the inner workings of the microbiome: genomic- and metagenomic-based study of metabolism and metabolic interactions in the human microbiome. *Cell Metab.* 20, 742–752. doi: 10.1016/j.cmet.2014.07.021

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3229–3236. doi: 10.1073/pnas.1218525110

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., et al. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature* 511, 224–227. doi: 10.1038/nature13491

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2013). *Package 'Vegan,' in: R Version 2.0-10*.

Ouellet, M., Mikaelian, I., Pauli, B. D., Rodrigue, J., and Green, D. M. (2005). Historical evidence of widespread chytrid infection in North American amphibian populations. *Conserv. Biol.* 19, 1431–1440. doi: 10.1111/j.1523-1739.2005.00108.x

Perez, R., Richards-Zawacki, C. L., Krohn, A. R., Robak, M., Griffith, E. J., Ross, J., et al. (2014). *Atelopus various* frogs are persisting in regions where *Batrachochytrium dendrobatidis* is now enzootic. *Amphibi. Reptile Conserv.* 8, 30–35.

R Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rebollar, E. A., Hughey, M. C., Harris, R. N., Domangue, R. J., Medina, D., Ibáñez, R., et al. (2014). The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of far eastern Panama. *PLoS ONE* 9:e95484. doi: 10.1371/journal.pone.0095484

Rollins-Smith, L. A., Reinert, L. K., and Burrowes, P. A. (2015). Coqui frogs persist with the deadly chytrid fungus despite a lack of defensive antimicrobial peptides. *Dis. Aquat. Org.* 113, 81–83. doi: 10.3354/dao02823

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., et al. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J.* 6, 564–576. doi: 10.1038/ismej.2011.116

Skaug, H., Fournier, D., Bolker, B., Magnusson, A., and Nielsen, A. (2014). *Generalized Linear Mixed Models using AD Model Builder in: R Package Version 0.8.0*.

Smirnov, N. (1936). Sur la distribution de ω2 (criterium de M. R. v. Mises). *Comptes Rendus l'Acad. Sci.* 202, 449–452.

Smith, K. G., Lips, K. R., and Chase, J. M. (2009). Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. *Ecol. Lett.* 12, 1069–1078. doi: 10.1111/j.1461-0248.2009.01363.x

Tibshirani, R. (1996). Regression shrinkage and selection via the LASSO. *J. R. Stat. Soc. B* 58, 267–288.

Umile, T. P., McLaughlin, P. J., Johnson, K. R., Honarvar, S., Blackman, A. L., Burzynski, E. A., et al. (2014). Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC fingerprinting. *Anal. Methods* 6, 3277–3284. doi: 10.1039/C4AY00566J

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., and Knight, R. (2012). Defining the human microbiome. *Nutr. Rev.* 70, S38–S44. doi: 10.1111/j.1753-4887.2012.00493.x

Venesky, M. D., Raffel, T. R., McMahon, T. A., and Rohr, J. R. (2014). Confronting inconsistencies in the amphibian−chytridiomycosis system: implications for disease management. *Biol. Rev.* 89, 477–483. doi: 10.1111/brv.12064

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2015). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *Appl. Environ. Microbiol.* 81, 6589–6600. doi: 10.1128/AEM.01486-15

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595. doi: 10.1890/14-1837.1

Woodhams, D. C., Kilburn, V. L., Reinert, L. K., Voyles, J., Medina, D., Ibáñez, R., et al. (2008). Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. *Ecohealth* 5, 268–274. doi: 10.1007/s10393-008-0190-0

Yildirim, S., Yeoman, C. J., Sipos, M., Torralba, M., Wilson, B. A., Goldberg, T. L., et al. (2010). Characterization of the fecal microbiome from non-human wild primates reveals species specific microbial communities. *PLoS ONE* 5:e13963. doi: 10.1371/journal.pone.0013963

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Spring-Verlag.

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