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MICROBIAL ECOLOGY

Differences in Temperature and Water Chemistry Shape Distinct Diversity Patterns in Thermophilic Microbial Communities

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ABSTRACT This report describes the biodiversity and ecology of microbial mats developed in thermal gradients (20 to 65°C) in the surroundings of three drillings (Chiraleu [CH], Ciocaia [CI], and Mihai Bravu [MB]) tapping a hyperthermal aquifer in Romania. Using a metabarcoding approach, 16S rRNA genes were sequenced from both DNA and RNA transcripts (cDNA) and compared. The relationships between the microbial diversity and the physicochemical factors were explored. Additionally, the cDNA data were used for *in silico* functionality predictions, bringing new insights into the functional potential and dynamics of these communities. The results showed that each hot spring determined the formation of distinct microbial communities. In the CH mats (40 to 53°C), the abundance of *Cyanobacteria* decreased with temperature, opposite to those of *Chloroflexi* and *Proteobacteria*. *Ectothiorhodospira*, *Oscillatoria*, and methanogenic archaea dominated the CI communities (20 to 65°C), while the MB microbial mats (53 to 65°C) were mainly composed of *Chloroflexi*, *Hydrogenophilus*, *Thermi*, and *Aquificae*. Alpha-diversity was negatively correlated with the increase in water temperature, while beta-diversity was shaped in each hot spring by the unique combination of physicochemical parameters, regardless of the type of nucleic acid analyzed (DNA versus cDNA). The rank correlation analysis revealed a unique model that associated environmental data with community composition, consisting in the combined effect of Na^+, K^+, HCO_3^-, and PO_4^{3-} concentrations, together with temperature and electrical conductivity. These factors seem to determine the grouping of samples according to location, rather than with the similarities in thermal regimes, showing that other parameters beside temperature are significant drivers of biodiversity.

IMPORTANCE Hot spring microbial mats represent a remarkable manifestation of life on Earth and have been intensively studied for decades. Moreover, as hot spring areas are isolated and have a limited exchange of organisms, nutrients, and energy with the surrounding environments, hot spring microbial communities can be used in model studies to elucidate the colonizing potential within extreme settings. Thus, they are of great importance in evolutionary biology, microbial ecology, and exobiology. In spite of all the efforts that have been made, the current understanding of the influence of temperature and water chemistry on the microbial community composition, diversity, and abundance in microbial mats is limited. In this study, the composition and diversity of microbial communities developed in thermal gradients in the vicinity of three hot springs from Romania were investigated, each having particular physicochemical characteristics. Our results expose new factors that could determine the formation of these ecosystems, expanding the current knowledge in this regard.

Received 20 June 2017 **Accepted** 11 August 2017

Accepted manuscript posted online 18 August 2017

Citation Chiriac CM, Szekeres E, Rudi K, Baricz A, Hegedus A, Dragoş N, Coman C. 2017. Differences in temperature and water chemistry shape distinct diversity patterns in thermophilic microbial communities. Appl Environ Microbiol 83:e01363-17. https://doi.org/10.1128/AEM.01363-17.

Editor Robert M. Kelly, North Carolina State University

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KEYWORDS hot spring, metabarcoding, microbial diversity, microbial ecology

Microbial mats are stratified communities of functional groups that are determined and maintained by steep physicochemical gradients (1). Hot spring microbial mats are of particular interest because they are considered model ecosystems for the study of early life on Earth. The oldest evidence of life on land came from the ca. 3.48-billion-year-old Archean rocks from Pilbara Craton, Western Australia. These structures include stromatolites, or mineralized microbial mats, that have developed in an ancient hot spring environment (2). Modern hot spring habitats, including various microbial mat ecosystems, have been extensively studied, especially in Yellowstone National Park in the United States (3–6), in the Tibetan Plateau, China (7–9), in Iceland (10, 11), and in Russia (12, 13). These studies have considerably improved our understanding of microbial composition and diversity in terrestrial hot spring ecosystems (7). Thus, it is known that these microbial mats lack seasonality and undergo changes in response to differences in physicochemical parameters, such as temperature, pH, and sulfide concentrations (1). These environments are dominated by prokaryotes, as extreme temperatures usually exclude eukaryotic organisms (14). Under hyperthermal conditions that are not suitable for photosynthesis (>75°C) (15), thermal and hyperthermal members of *Bacteria* are common inhabitants of microbial mats, being predominantly affiliated with the *Aquificae*, *Thermatogae*, and *Deinococcus-Thermus* (*Thermi*) phyla or with certain thermophilic members of *Proteobacteria* (7). At lower temperatures and under light conditions, oxygenic and anoxygenic forms of photosynthesis usually support the primary production in the mat, allowing a diversification of the microbial communities (16). Photosynthetic hot spring microbial mats may be sustained by *Cyanobacteria*, *Chloroflexi*, or *Chlorobi* or by a combination of these groups (17). Besides the bacterial domain, *Archaea* are also present in hot spring mats, being represented especially by the *Crenarchaeota* and *Euryarchaeota* phyla (18, 19).

To better understand the ecology and microbial functions leading to the establishment of microbial mats, a number of studies have focused on the links between the physicochemical parameters of the water and the microbial composition and diversity (7, 11, 16, 20–22). Among those factors, temperature has received much attention. While some studies have explored the temperature effect on small-scale gradients (16, 20), others have selected springs within a wide temperature range (11, 23). The results showed that the microbial mat composition and the complexity of the ecosystem seem to be mainly determined by the rise in temperature (1). In a study performed by Wang et al. (7), mat samples were collected from thermal gradients in different areas and no location-specific variations were observed. Instead, the temperature was sufficient to explain the variability in community composition. In contrast, other studies found that the sulfide concentration (21) and the pH value (24) were important factors that control the similarity among samples. Nevertheless, more effort is required to better understand the effects of physicochemical conditions on the development of hot spring microbial ecosystems.

As a result of geothermal resource exploitation in the second half of the 20th century, eight geothermal areas were identified in Romania, with over 200 drillings ranging in depth from 800 to 3,500 m and having outflow water temperatures from 40°C to 120°C (25). Among these, a number of drillings (80) tap the Pannonian hyperthermal aquifer (50 to 85°C) from the Western Plain of Romania, the water that comes from 800 to 2,100 m deep being used mostly for domestic heating, health and recreational bathing, industrial processes, and fish farming (25). Some wells have been abandoned over the years, and the continuous hot water flow has led to the development of specific mineralized communities and other photosynthetic microbial mats (26, 27). Even though the biodiversity in some of these microbial mats has been previously described using 16S rRNA gene clone libraries (26), the current study applied high-throughput sequencing techniques for a more thorough investigation. It focused on microbial mats formed along thermal gradients in hot springs from three different

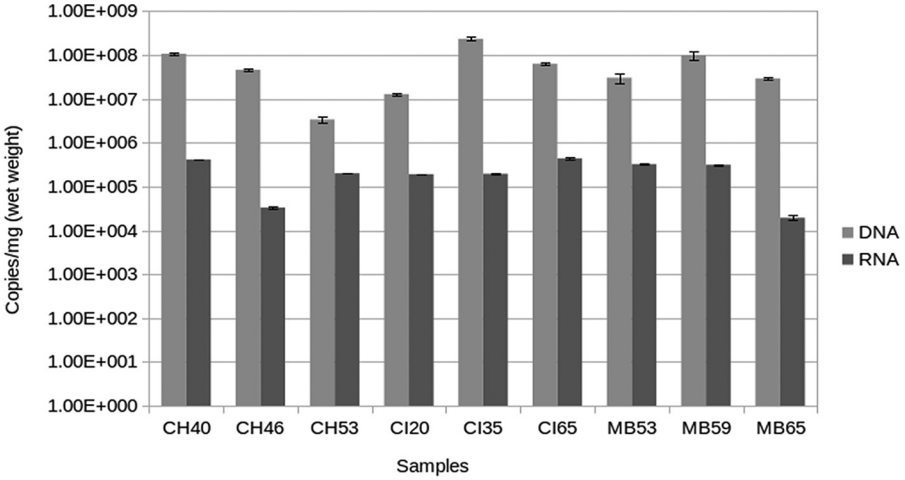

FIG 1 Quantification of 16S rRNA gene and transcript abundances by qPCR in each of the investigated hot spring microbial mats.

locations, each with particular physicochemical characteristics. An integrative approach was performed that included both 16S rRNA transcripts and 16S rRNA gene sequencing. Although the amount of rRNA that is detected in a microorganism is no longer viewed as a clear indicator for microbial growth, a large number of copies does indicate the potential for protein synthesis, for a rapid shift in metabolic function, or for an overall higher fitness of that species in the ecosystem (28). The inferences of metabolic potential based on 16S rRNA transcripts may fall under some degree of uncertainty (29). But taking into consideration that extracellular RNA is less stable in the environment than free DNA (30), functional roles can be attributed in this way to intact organisms. In contrast, the DNA extracted from environmental samples is a continuum of DNA derived from whole living organisms and extraorganismal, free DNA (31). A more precise view on the microbial community composition, its diversity, and its function may be proposed by comparing the 16S rRNA gene libraries with those of 16S rRNA transcripts.

Thus, the aim of this research was to characterize the prokaryotic composition and biodiversity in the newly investigated hot springs microbial mats associated with three hot springs situated in the Western Plain of Romania and to explore their relationship with the physicochemical properties of the thermal water. As these microbial communities may bring new information on the distribution of microbial groups in thermal environments, an additional effort was made to compare their microbial compositions with those of other microbial mats that have been previously investigated through a metabarcoding approach.

RESULTS AND DISCUSSION

Abundance of prokaryotic 16S rRNA genes and transcripts. The abundance of prokaryotic microorganisms in the DNA and cDNA samples was estimated through quantitative real-time PCR (qPCR) by targeting the 16S rRNA gene. Generally, the copy numbers in the cDNA transcripts were found to be 2 to 3 orders of magnitude lower than the numbers observed in the DNA sample (Fig. 1). This difference may suggest that evaluation of cell abundances should not be based solely on the 16S rRNA genes. Some studies have proposed that up to 90% of the free DNA is stable in sediments and may not be affected by nucleases (32, 33). Thus, the total extracted DNA most probably reflects not only the living microbes present in the environment but also the extracellular DNAs (34). Considering the instability of the free RNA, it seems that it could provide a better estimation of the actual abundance of microbial cells that can synthesize proteins (1, 35). In the Chiraleu (CH) samples, the number of 16S rRNA gene copies decreased with temperature from $1.1 \times 10^8 \pm 0.01 \times 10^8$ at 40°C to 3.4 ×

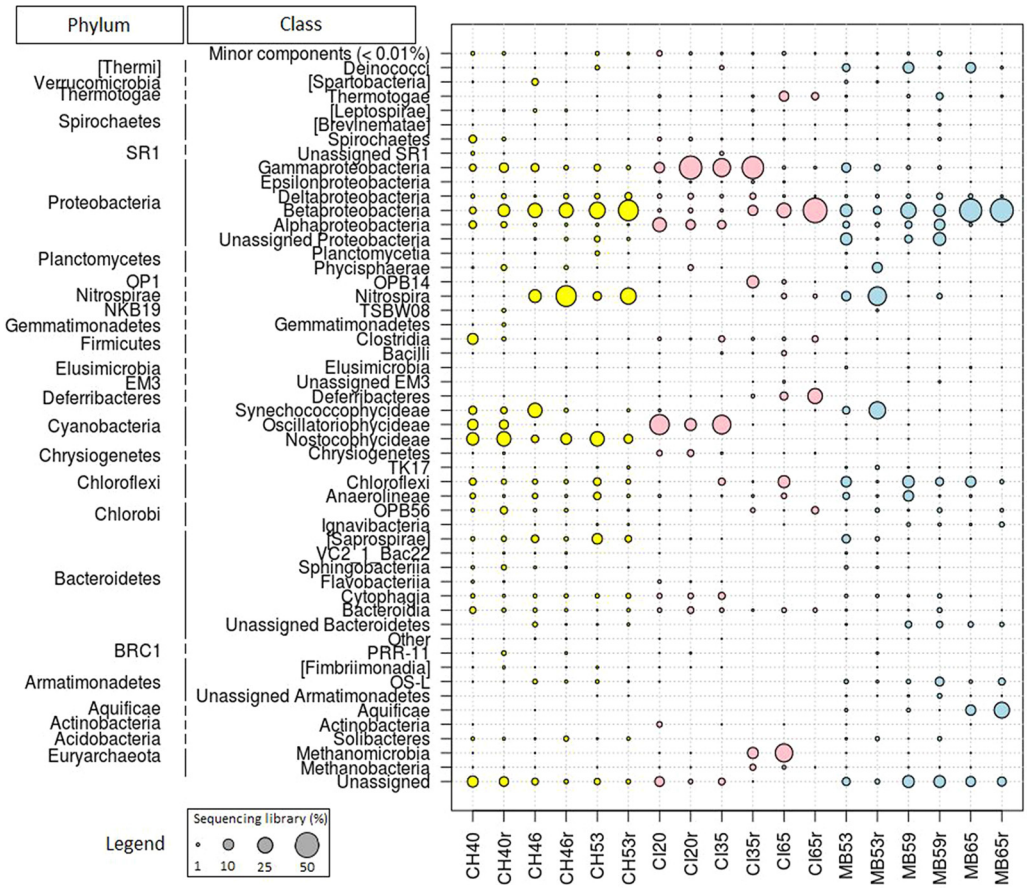

FIG 2 Relative abundances of prokaryotic classes and phyla encountered in the hot spring microbial mats. The sequencing results from both the DNA- and cDNA-based approaches are shown side by side. Different colors are used to represent the three sampling sites: yellow, Chiraleu (CH); pink, Ciocaia (CI); and blue, Mihai-Bravu (MB).

$10^6 \pm 0.6 \times 10^6$ copies/mg at 53°C, whereas at the other sampling sites no clear pattern was observed. In the Ciocaia (CI) DNA samples, the abundances of 16S rRNA genes ($1.2 \times 10^7 \pm 0.05 \times 10^7$ to $2.3 \times 10^8 \pm 0.27 \times 10^8$ copies/mg) were comparable with those reported in a previous study focused on the microbialites described at temperatures between 32 and 65°C in the proximity of the currently investigated microbial mats (27). The copy numbers of 16S rRNA in the Mihai Bravu (MB) samples ($2.89 \times 10^7 \pm 0.1 \times 10^7$ to $9.9 \times 10^8 \pm 0.2 \times 10^8$ copies/mg) were similar to those in the other two locations, and no direct relationship with temperature was observed. As in the case of rRNA genes, the 16S rRNA copy number in the cDNA samples did not show a monotonic relationship with temperature fluctuations in any of the investigated hot springs (Pearson $r_{cDNA} = 0.0921$; $r_{DNA} = -0.211$; $P > 0.05$) (Fig. 1). Whereas a linear relationship was reported in the literature between the abundance of specific microbial groups and the increase in temperature, this was not the case for the samples within this study. Hence, more effort is needed to examine the effect of temperature on the overall microbial abundance (7).

Microbial communities revealed by 16S rRNA gene sequencing. Following raw sequence quality filtration, a total of 498,539 sequences were kept in the final data set that included both the 16S rRNA gene and 16S cDNA libraries. Each sample was represented by 3,499 to 78,280 high-quality sequences that were clustered at a 97% identity threshold in 639 operational taxonomic units (OTUs). Figure 2 summarizes the phylum and class level compositions of samples; it shows that overall, up to 13.5% of the sequencing libraries were unassigned, underlining once again the importance of such extreme environments in the discovery of novel microbial groups with unknown

features. *Archaea* were present in all samples except MB65r, being represented mainly by aceticlastic methanogens from the *Methanosaeta* (31.6%) genus in CI65, together with *Methanoculleus* (5.3%) and *Methanothermobacter* (3.2%) in CI35r. Regarding the bacterial taxonomic composition, the groups with the highest abundances were *Proteobacteria* (13.2 to 66.2%), *Cyanobacteria* (up to 40.3%), *Chloroflexi* (up to 24%), *Bacteroidetes* (up to 12.8%), and *Firmicutes* (up to 11.3%). The remaining sequences were affiliated widely across the *Bacteria* domain, in the *Deinococcus-Thermus* (*Thermi*) (5.7 to 11.2%), *Thermatogae* (up to 9.03%), *Nitrospirae* (2.6 to 15.75%), *Spirochaetes* (up to 5.94%), *Verrucomicrobia* (up to 4.32%), and *Aquificae* (up to 10%) phyla.

CH hot spring microbial mats. The CH samples were mainly dominated by *Proteobacteria* and *Cyanobacteria* (Fig. 2). The abundance of *Proteobacteria* increased with temperature, with an inverse relationship being observed for the oxygenic phototrophic group (Fig. 2). While photosynthesis can take place at temperatures up to 75°C (15, 36), distinct taxonomic groups are adapted to perform this function at different concentrations of oxygen, at variable temperatures, or at variable light intensities by using various electron acceptors and donors (37). Moderately thermophilic cyanobacteria from the genera *Leptolyngbya* and *Oscillatoria* and from the order *Nostocales* dominated the oxygenic mats at 40 and 46°C and were most probably the primary producers (1). In contrast, the *Chloroflexus* genus became dominant at 53°C (Fig. 2; see also Fig. S1 in the supplemental material), members of this group being able to assemble a fully functional photosynthetic apparatus under anoxic conditions. Such anoxic conditions may be caused by the increased water temperature, which lowers the solubility of various gases, including oxygen (38). In the absence of O_2 or under fluctuating oxic/anoxic circumstances, and in the presence of H_2 and H_2S, *Chloroflexus* may grow photoautotrophically by assimilation of CO_2 through the 3-hydroxypropionate pathway (39). In some cases, *Cyanobacteria* and *Chloroflexi* may compete for limited resources (40), but their association was previously documented under thermal conditions, where the oxygenic photosynthesis was dependent on sulfide consumption by *Chloroflexus* species (41, 42). As *Cyanobacteria* possess chlorophyll *a* (680 nm) and *Chloroflexi* synthesize bacteriochlorophylls *c* and *a* (740 nm and 790 nm), they are able to coexist at harvesting different wavelengths of light (37, 43). Thus, it can be assumed that the two groups form distinct assemblages in the mat layers, and both can contribute to the primary production of the ecosystem (37).

Even though the members of the *Methylophilaceae* family (*Betaproteobacteria*) are usually restricted to use methanol or methylamine as carbon sources and are common inhabitants of mud, lake and pond water, and sludge (44), this group makes up almost 8% of the CH46 library. Although not typical for a hot spring environment, members of this family were previously reported to occur in wet sediments collected from Magadi Lake (Kenya), a hypersaline lake that is supplied by a series of thermal springs with temperatures up to 86°C (45), and in a pond from the Kirishima geothermal area, Japan (46). Another abundant phylum in the CH microbial mats was *Nitrospirae* (sample CH53, 7%, and sample CH46, 15.75%), represented mainly by the *Nitrospira* genus. It seems that the moderately thermophilic conditions from CH create an environment that is suitable for nitrifying organisms such as *Nitrospira*, a widespread group that thrives in habitats with temperatures below 55°C in Europe, Asia, and Australia (13).

CI hot spring microbial mats. Microbial mats which developed at lower temperatures (20 and 35°C) near the CI hot spring were represented by almost equal proportions of *Cyanobacteria* (34.2 to 40.4%) and *Proteobacteria* (32.2 to 40.7%) (Fig. 2). *Bacteroidetes* was the next most abundant group (5.6 to 6.5%) in both samples. While in sample CI20 *Actinobacteria* (2.8%) and *Chrysiogenetes* (2.9) were more abundant, in sample CI35 higher abundances of *Chloroflexi* (5.5%) and *Firmicutes* (4.2%) were detected.

Almost all cyanobacterial sequences in these two samples were affiliated with the *Oscillatoria* genus and showed a decrease in abundance with an increase in water temperature (see Fig. S1). Usually, *Oscillatoria*-dominated microbial mats tend to have

a limited distribution, with communities from higher temperatures being usually formed by the *Pseudoanabaena*, *Synechococcus*, and *Chloroflexus* genera (47). Microbial mats dominated by *Oscillatoria* spp. were found in the waters from the Chocolate Pots hot springs (36 to 45°C) (Yellowstone National Park, USA), rich in iron, bicarbonate, and silica (48). In mesothermal and saline environments, nitrogen-fixing members of *Oscillatoria* were found to be the first in colonizing new surfaces, as in the case of the microbial mats from the Mellum island in the North Sea and in the Laguna Figueroa (Pacific Ocean) (37, 49). Also, *Oscillatoria boryana* formed a predominant population in the mat developed around a sulfide-rich geothermal spring in Rotorua (New Zealand) (50).

Within *Proteobacteria*, the *Alphaproteobacteria* and *Gammaproteobacteria* classes prevailed in samples CI20 and CI35. *Salinarimonas rosea* and *Ectothiorhodospira* spp. were the most dominant taxa in sample CI20, and both have been previously encountered in hot spring microbial mats. The first one is a halotolerant, facultatively anaerobic bacterium isolated from a salt mine in Yunnan (China) (51) and was also encountered in a phototrophic microbial mat from Hot Lake, WA (52). The second taxon includes purple sulfur bacteria that may form a red layer in microbial mats under the oxic, green layer dominated by *Cyanobacteria* (37, 49). Like all purple sulfur bacteria, *Ectothiorhodospira* members are capable of photosynthesis, using hydrogen sulfide or monothioarsenate instead of water as an electron donor, oxidizing them to elemental sulfur or arsenate, respectively (53, 54). Because the synthesis of bacteriochlorophyll in these microorganisms is inhibited in the presence of oxygen and under high light intensities, they are found in mats developed in the photic zone of aquatic environments at the oxic-anoxic interface (55). Similar hot spring biofilms composed primarily of *Oscillatoria*-like and *Ectothiorhodospira*-like organisms were previously described for Mono Lake, CA (56).

Other microorganisms with a high abundance in the sample collected at 35°C were included in the *Chloronema* genus (5% of the sequencing library) of the *Chloroflexi* phylum. This is a surprising observation since these anoxygenic filamentous phototrophs usually require low temperatures (4 to 15°C) for growth (57). The presence in CI35 of *Chloronema* members, which can tolerate higher temperatures than those previously reported in the literature, may indicate the existence of a new taxon within this genus. The *Flexibacter* genus was also dominant (4.5%), some species (i.e., *Flexibacter flexis*, *F. roseolus*, and *F. ruber*) being common for hot spring environments (58, 59).

Sample CI65 comprised 101 OTUs with highly uneven proportions. The *Methanosaeta* genus comprised 31.6% of the library. This group, even though it is usually encountered in anaerobic sediments and sewage sludge digesters (60), has also been detected in other thermal settings, such as the geothermal waters of the Sungai Klan spring (Malaysia) (61) and in an alkaline hot spring from Iceland (62). While in the other CI samples *Gammaproteobacteria* were dominant, it seems that they were replaced by *Betaproteobacteria* at 65°C. The second-most abundant OTU (18.5%) was affiliated with the *Hydrogenophilus* genus of *Betaproteobacteria*, a group of thermophilic and aerobic organisms that can have a facultatively chemolithoautotrophic metabolism, using H_2 as an electron donor and CO_2 as a carbon source (63).

MB hot spring microbial mats. The microbial mats from MB were encountered at the highest temperature range, from 53 to 65°C. In these microbial mats, *Cyanobacteria* were present as a major group only at 53°C (5.4%) (Fig. 2). It seems that the environmental conditions favor the *Chloroflexi* phylum, which rose from 15% in MB53 and MB65 to 24% of the library at 59°C. In contrast, the proportion of *Betaproteobacteria* (*Rhodocyclales*) increased with temperature (see Fig. S1) and was mostly represented by *Hydrogenophilus* members. These results imply that the MB mats may be sustained either by anoxigenic photosynthesis, by chemolithoautotrophy through the oxidation of hydrogen, or by both of these strategies. Two particular and dominant phyla in MB samples are *Thermi* (*Deinococcus-Thermus*) (5.7 to 11.2%) and *Aquificae* (10%). Both of

these phyla are collections of bacteria adapted to harsh and extreme environmental settings (64, 65). *Aquificae* representatives became abundant only in sample MB65, as they are strictly thermophiles, with an optimum growth temperature above 65°C (66). They have been encountered in volcanic and geothermal heated environments, such as the hot ponds in Iceland, Yellowstone National Park, USA (67), and Tuscany, Italy (68), or in neutral and acidic hot springs in Philippines (69). The vast majority of these organisms are obligate autotrophs, using hydrogen and carbon dioxide as electron and carbon sources and oxygen as the final electron acceptor (70). Moreover, the wide distribution of *Aquificae* may indicate that these bacteria have an important role in high-temperature habitats as primary producers (67, 70).

The *Meiothermus* and *Thermus* genera within the *Thermi* phylum were dominant members of the MB mats. The different optimal growth temperatures for the two taxa may explain why the *Meiothermus* proportion was higher in samples MB53 and MB57, whereas *Thermus* prevails at 65°C. A similar trend, a decrease of *Meiothermus* abundance with temperature and an increase of *Thermus* proportion, was observed in microbial mats colonizing an aquifer bore runoff channel from Australia (71). They are commonly encountered genera in hot springs with moderate to high temperatures (50 to 99°C) and slightly alkaline or acidic waters, as those from Kamchatka (72), Yellowstone National Park (73), or Thailand (21). Although these microorganisms do not usually become major components in surface environments, situations similar to those in MB mats have been encountered in several other springs in Iceland and the Tibetan Plateau, China (7, 11).

Indicator species in the hot spring microbial mats. As previously mentioned, 639 OTUs were observed in the microbial mats collected from the three distinct locations. A multilevel-indicator species analysis (see Table S2 in the supplemental material) revealed that only a few of them (<10) were particular for each sampling location and that the vast majority of OTUs were shared among hot springs, even though their abundances varied from site to site. In both sequence libraries from the CH hot spring, members affiliated with the orders *Nostocales* and *Thiobacterales* and with the genera *Chloroflexus* and *Hydrogenophilus* were detected as specific for this hot spring. Among the dominant OTUs in the CI microbial mats, the indicator taxa for this hot spring were *Ectothiorhodospira*, *Desulfurispirillum*, *Fervidobacterium*, *Methanothermobacter*, and *Nitrincola*. Even though the MB mats had the lowest number of observed OTUs, they had the largest amount of indicator species, including the genera *Chloroflexus*, *Thermus*, and *Meiothermus* and one unassigned OTU (99% similarity with the sequence having accession number FR691784 in the GenBank database) that has been previously encountered in a slightly alkaline, sulfur-metabolizing microbial mat from the Nakabusa hot spring in Japan (74). Although this taxon is yet unaffiliated with any phyla within the *Bacteria* domain and nothing is known about its functional role, it constituted ~10% of samples MB59 and MB59r and is possibly an important member in similar ecosystems from various geographic areas. Additionally, two OTUs were affiliated with the *Hydrogenophilus* genus; one of them was much more abundant, making up to half of the communities in the MB and CI samples, while the other one was particular for the CH libraries.

Comparative analysis of DNA and cDNA libraries and putative functional traits of microbial mats. Many studies have conducted comparative metabarcoding analyses, focusing on the 16S rRNA genes (DNA-based library) or rRNA transcripts (cDNA-based library) (45, 75, 76), targeting the observed versus active microbial communities. This approach is feasible, as extracellular RNA is usually unstable in the environment, and its proportions are in some cases correlated with the growth rate in a variety of bacteria (35). Therefore, this approach can be used to estimate the active members of the community. The downfall is that for the vast majority of microorganisms, there is a lot unknown regarding the relationship between the cell size, life stages, dormancy, the number of *rrn* operons, and ribosomal content (35). Therefore, a precise contribution of specific microorganisms to the entire community cannot be determined with

certainty by either of the two methods. Nevertheless, Blazewicz et al. (28) pointed out that even though 16S cDNA data are not necessarily informative on the actual occurring protein synthesis, the data can show the potential limitation of protein synthesis for a population at a given time. Additionally, changes in the apparent ribosomal content may be influenced by the RNA extraction protocol, since not all microbial cells are lysed with the same efficiency (35). Considering all these aspects, discussing the microbial populations and their dynamics based on the comparative analysis between DNA and cDNA data should be carried out with caution (28).

For the samples analyzed in this study, the DNA-cDNA comparison showed important changes in relative abundances of several groups within the *Cyanobacteria*, *Proteobacteria*, *Nitrospira*, *Aquificae*, and *Methanomicrobia* phyla (see Fig. S2 in the supplemental material). In looking at the microbial mats from CH and CI, decreases in the abundances of *Synechococcophycideae* (−18.5%), *Nostocophycideae* (−12.6%), and *Oscillatoriophycideae* (−34.2%) sequences were observed in the cDNA libraries. As previously mentioned, this aspect does not necessarily imply a reduction in the population size; rather, it implies a reduction in the protein synthesis activity in *Cyanobacteria*, as Blazewicz et al. (28) concluded that the amount of rRNA is to be considered "a reflection of the past, current and future activities." Lepp and Schmidt (77) showed that during the dark diurnal period, *Synechococcus* populations synthesize and accumulate rRNA, which will confer an advantage under favorable light conditions; the same adaptation has been observed in a *Prochlorococcus* strain as well (78). Even though it is only an assumption, some anticipatory strategies may also be present in the metabolism of *Oscillatoriophycideae* members and other *Cyanobacteria*, but this aspect needs further investigation. On the assumption that the taxa revealed by the 16S rRNA sequencing library are potentially capable of protein synthesis in the microbial mats (28), the putative metabolic pathways and the relative abundance of the genes involved in these processes were predicted using PICRUSt software (79). The highest variations were observed in the case of genes responsible for photosynthesis (see Fig. S3 in the supplemental material), probably as a reflection of high *Leptolyngbya* and *Chloroflexus* populations in samples CH40r and MB53r, whereas *Oscillatoria* presented a significant decrease in the CI20r and CI35r mats compared with their rRNA gene libraries.

Regarding the proteobacterial ribosomal content in relation with the cellular growth and activity, Kerkhof and Kemp (80) showed that no singular trend can be generally applied to this phylum. Some of the proteobacterial groups presented an indirect or direct linear correlation between the two variables; others had no apparent relationship. When Wüst et al. (35) investigated the rRNA/rRNA gene ratio in soil samples, they reported a 3-fold increase in *Deltaproteobacteria* and *Betaproteobacteria*, concluding that most probably these groups contain large numbers of active individuals. *Betaproteobacteria* and *Gammaproteobacteria* had high rRNA/rRNA gene ratios in the CI microbial mats, mainly represented by increases of the *Ectothiorhodospira* (CI20r) and *Hydrogenophilus* (CI65r) genera. The *Ectothiorhodospira* genus brings together typical phototrophic sulfur bacteria (81), while *Hydrogenophilus* encompasses predominantly chemoautotrophic thermophiles (63). Regarding the carbon fixation pathways in prokaryotes, sample CI65r showed high relative abundances of genes possibly involved in performing this activity (see Fig. S3). Considering these aspects, it may be assumed that both genera are potentially important contributors to the primary production in these microbial ecosystems. *Fervidobacterium* (6.4%) was also present in CI65r, and a syntrophic relationship between its members and the *Hydrogenophilus* population can be speculated. While *Hydrogenophilus* is able to use H_2 as an electron donor, members of *Fervidobacterium* produce important quantities of H_2 through the fermentation of organic compounds. Thus, for this dense packed microbial ecosystem, a cyclic electron flow can be proposed (63, 82).

Surprisingly, the *Metanosaeta* genus was the most abundant OTU in sample CI65 (31.6%) but was absent in CI65r (Fig. 2). Accordingly, methane metabolism did not seem particularly dominant in CI65r (see Fig. S3). Either this is a possible result of the RNA

TABLE 1 Physicochemical characteristics of the hot spring waters[a]

Parameter	Value for:		
	Chiraleu	Ciocaia	Mihai-Bravu
Na^+ (mg/liter)	676	4,120	931
K^+ (mg/liter)	16.5	99.2	13.3
Ca^{2+} (mg/liter)	4.53	10.2	15
Cl^- (mg/liter)	1,160	820	537
HCO_3^- (mg/liter)	1,140	1,980	895
CO_3^{2-} (mg/liter)	96	<6	132
SO_4^{2-} (mg/liter)	2.7	18.9	50
NH_4^+ (mg/liter)	23.2	25	0.09
Fe^{2+} (mg/liter)	0.136	0.114	0.383
Mn^{2+} (mg/liter)	0.008	0.004	0.018
NO_3^- (mg/liter)	0.59	0.81	1.08
PO_4^{3-} (mg/liter)	120	390	150
pH	7.86	7.75	7.91
Water temp (°C)	57	67	60
Water flow (liters/s)	4	3.5	4.5
Conductivity (μS/cm)	2,570	12,100	2,780

[a]SO_4^{2-} data are from reference 92; all other data are from reference 91.

extraction bias or these microorganisms were carried by the water flow from the subsurface but were not able to establish populations in the microbial mats. Because this genus encompasses acetotrophic methanogens that need acetate as a substrate for growth (83), it is possible they were outcompeted in this sample by other organisms that use acetate as an electron donor, like *Defferibacter* (21.8%) (84). Instead, other methanogens included in the *Methanothermobacter* (3.2%), *Methanosaeta* (6.06%), and *Methanoculleus* (5.3%) genera are major components in CI35r, which is reflected by the PICRUSt results as well (see Fig. S3). Besides *Methanosaeta*, the other two genera perform hydrogenotrophic methanogenesis (85, 86) and seem to be able to coexist with a small population of *Hydrogenophilus* (5.6%), although all of them require H_2 for growth.

MB hot spring waters are different from those of CH and CI due to a high concentration of sulfate (50 mg/liter [Table 1]). Additionally, the *Aquificae* phylum appears abundant in sample MB65, and it was even more prevalent in the cDNA data. Investigating some Icelandic hot springs, Skirnisdottir et al. (87) reported that high-sulfate springs with temperatures below 70°C were commonly dominated by *Aquificae*, while low-sulfate waters had higher abundances of *Chloroflexus* spp. This may be also valid in the case of MB65r, in which *Aquificae* made up over 24% of the sequencing library, while *Chloroflexi* were restricted to only 1.5%. In this regard, the PICRUSt results for MB65r show a more intense sulfur metabolism (see Fig. S3). Based on the NSTI scores of 0.161 to 0.197 (see Table S1), the quantitative functional predictions for the MB samples are less accurate than those for the other two hot springs. These results may be explained by the low number of annotated genomes that are closely related to the OTUs observed in the MB microbial mats (79). Moreover, the comparative analysis also shows that several taxonomic groups involved in the nitrogen cycle are common in the cDNA samples, performing NO_2^- oxidation (*Nitrospira*), NO_3^- oxidation (*Nitrosomonas*), denitrification and assimilation (*Rhodospirillaceae*), and N_2^- fixation (*Chlorobi* and *Cyanobacteria*). Indeed, our analysis indicates that robust geochemical cycling processes are taking place in the microbial mat ecosystems, and it provides new insights into the community potential and dynamics issues. Nonetheless, future biochemical studies remain essential for the characterization of the true metabolic diversity in these microbial mats.

Alpha- and beta-diversity patterns. The microbial richness and diversity indices (i.e., Shannon, PD-whole tree, Chao1, and the total number of observed OTUs) were all negatively correlated with the increase in temperature (Pearson r between -0.708 and -0.468; $P < 0.05$). The Simpson diversity index was an exception, as the correlation with temperature (Pearson $r = -0.196$) was not significant at a P value of <0.05. Similar

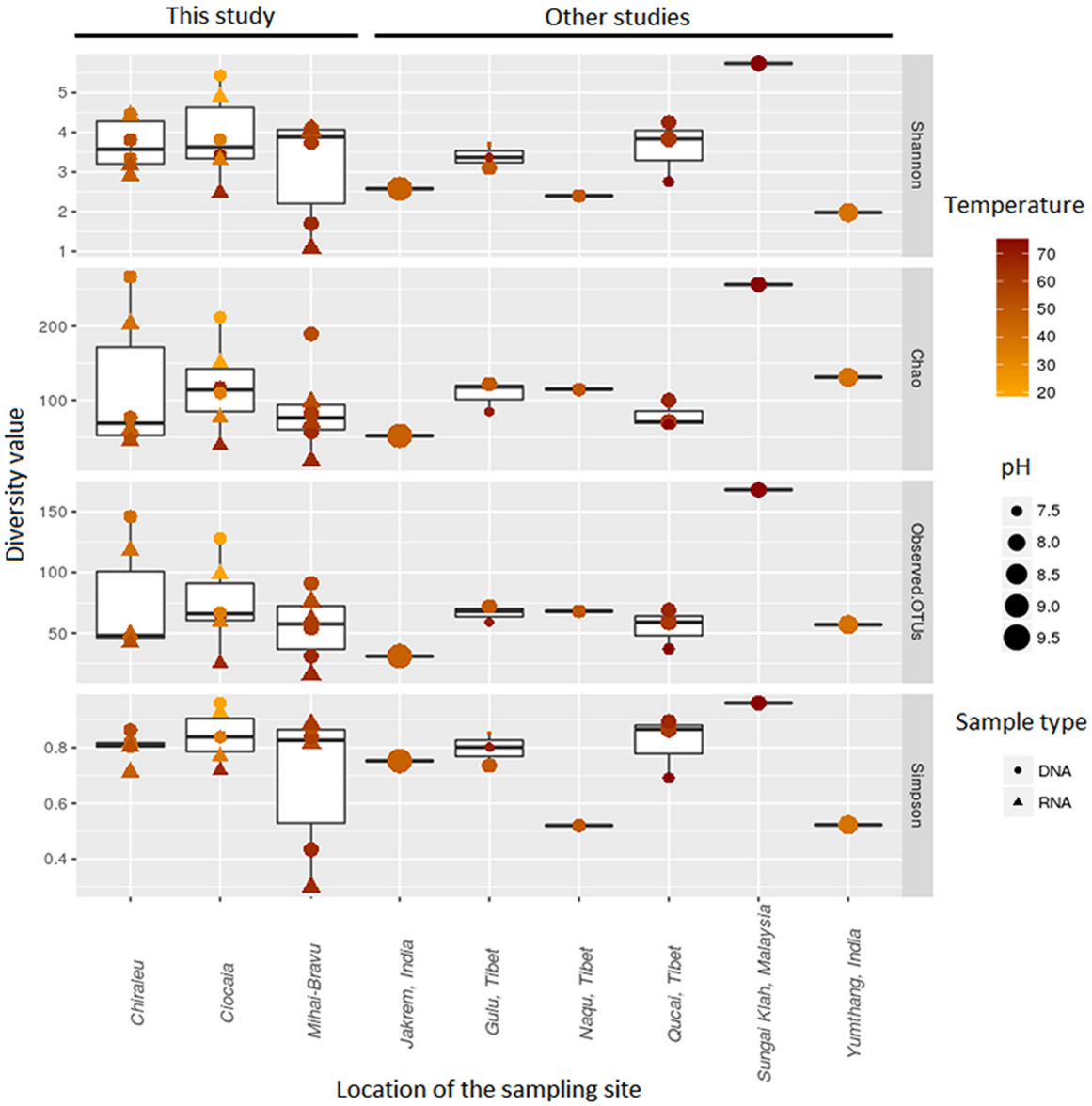

FIG 3 Shannon and Simpson diversity indices, the Chao1 richness estimator, and the number of observed OTUs in the samples from Chiraleu, Ciocaia, and Mihai Bravu, together with other microbial mats from similar sites (China, SRX206469, SRX206467, SRX206466, SRX206468, SRX206459, SRX206460, and SRX206456 [7]; India, SRS932137 and SRS932073 [89]; and Malaysia, PRJEB7059 [61]). Colors show the temperature measured at each sampling site, the shapes correspond to different nucleic acids, and the sizes are proportional to the pH values.

results, showing a decrease in alpha-diversity with temperature, were previously reported for hot spring samples from Iceland (11), Japan (16), and Yellowstone National Park, USA (6). This is not surprising, as fewer species manage to adapt when the environments become more extreme (88). Even though the environmental conditions that influence the biodiversity and microbial interactions in hot spring habitats are poorly understood (7), temperature is generally believed to be strongly involved in shaping the microbial composition and the diversity patterns (7, 11, 16, 40). Thus, we compared the alpha-diversity results from this study to those for other hot spring microbial mats reported in the literature and those whose methodological approach allowed inclusion in this analysis (Fig. 3). Overall, the number of OTUs in the majority of the DNA samples was higher than in those from the cDNA library (Fig. 3; see also Fig. S4 in the supplemental material), which may reflect the environmental accumulation of free DNA (34). Interestingly, the microbial mat from Sungai Klah, Malaysia, had the

highest diversity values even though it was collected from the most extreme investigated temperature (75°C) (61). In contrast, much lower values were obtained for a microbial mat collected at the same temperature in Qucai, China. In this case, the results were similar to those for samples CH46 and CH46r and to those from Jakrem, India (46°C) (89). These results highlight the need for extensive investigation on the microbial diversity issue using other environmental factors. For the microbial mats described in this study, the highest species richness, evenness, and phylogenetic diversity were observed in samples CI20, CH46, and MB53, whereas the lowest diversity was encountered in CI65r and MB65r, with only 25 and 16 observed OTUs, respectively.

In order to investigate the beta-diversity among the hot spring microbial mats included in this study, we tested which methodological approach of 16S rRNA gene sequencing (DNA versus cDNA) and which specific conditions from each of the three locations (CH, CI, and MB) were more significant in determining the observed similarities. To clarify this aspect, an analysis of similarity (ANOSIM) with 999 permutations was run for each of the two hypotheses, and the results showed that the sampling sites, with their own physicochemical characteristics, were responsible for the development of particular microbial communities ($r_{Location} = 0.343$ and $P = 0.001$; $r_{DNA/cDNA} = 0.061$ and $P = 0.136$). These results imply that even if some fluctuations in the abundance of particular taxa may be observed when comparing DNA and cDNA microbial diversity in the samples investigated, the overall communities remain similar, regardless of the type of nucleic acid analyzed, and the community composition is shaped by the combination of physicochemical parameters of each sampling site. This finding was further confirmed by a standard Mantel test performed for the physicochemical data and the weighted Unifrac dissimilarities (Mantel $r = 0.4313$ and $P = 0.001$), revealing that the beta-diversity was directly correlated with the environmental factors. For a better understanding of the effects of specific physicochemical parameters, and to quantify the relative contribution of each variable and combinations of variables on the microbial diversity, a rank correlation analysis was performed on the weighted Unifrac matrix. The best model that correlated the environmental and community data consisted in a subset of six environmental parameters, including Na^+, K^+, HCO_3^-, PO_4^{3-}, temperature, and electrical conductivity (Spearman's rho = 0.456).

To visualize and better understand the beta-diversity of the investigated microbial mats, weighted and unweighted Unifrac dissimilarities were chosen for principal-coordinate analysis (PCoA) ordination (Fig. 4). These matrices provide the highest explanatory values for the observed variability in the microbial communities (see Table S3), suggesting that the fluctuations in taxon abundances, together with their phylogenetic spread, play an important role in describing the beta-diversity. The results of ordination using weighted Unifrac dissimilarities (Fig. 4a) showed that the MB microbial mats, characterized by the highest and most similar temperatures (53 to 65°C), clustered together and thus were less variable. The opposite seems to be the case of the CI samples, with microbial mats formed at a wider range of temperatures (20 to 65°C). As previously supported by the rank correlation analysis, the strongest correlation of beta-diversity is not with temperature but with other ions that are present in the hot spring waters, such as Na^+, K^+, HCO_3^-, and PO_4^{3-} (Fig. 4). The influence of temperature on the microbial diversity was assessed in numerous studies (16, 20, 40, 90) that collected samples either along a thermal gradient (16, 20, 90) or from different hot springs with a wide range of temperatures (11). Some concluded that temperature was the most important factor and was sufficient to explain the microbial community composition (7), but other studies (21, 23) have shown that a combination of temperature and geochemistry factors (e.g., pH and sulfide) contributed the most in explaining the beta-diversity. In our case, temperature was indeed a major factor that influenced the observed diversity pattern, but the microbial distribution was better explained only when a combination of environmental ions was also included in the analysis. In the unweighted Unifrac PCoA (Fig. 4b), microbial mats appear to be loosely clustered according to sampling sites. This distance matrix takes into account only the presence or absence of OTUs, regardless of their abundances, as it tends to emphasize the

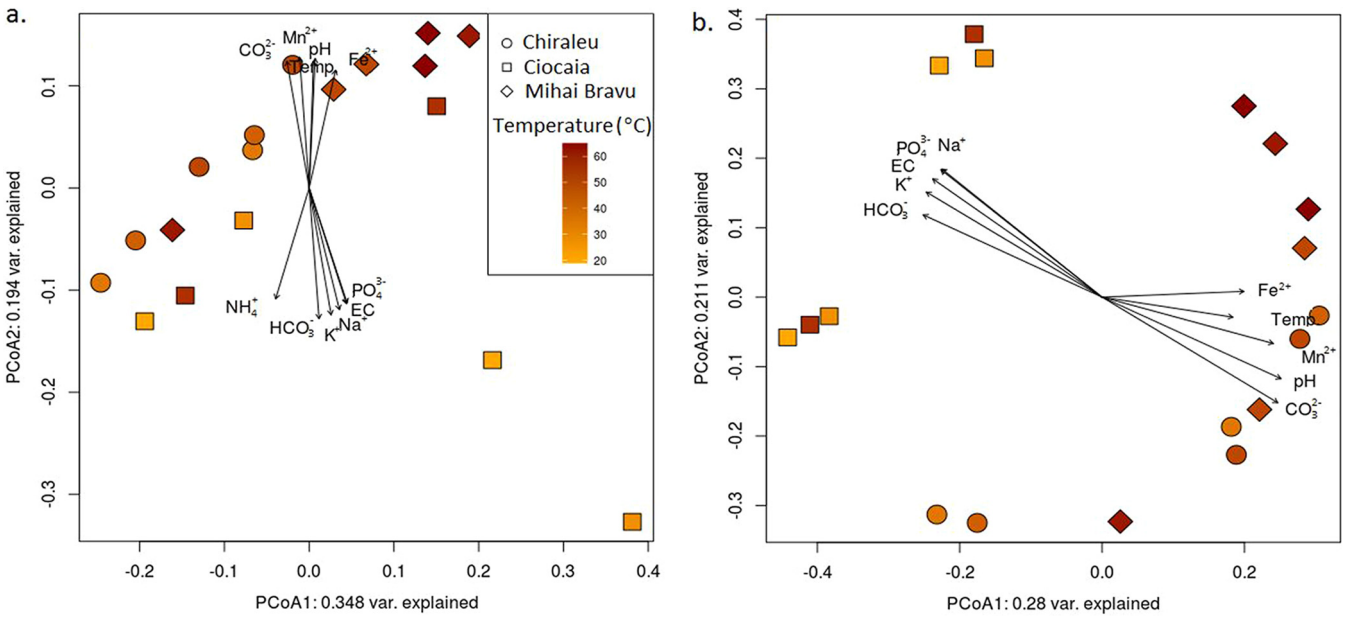

FIG 4 Principal-coordinate analysis (PCoA) based on weighted (a) and unweighted (b) UniFrac distances of microbial community structure. The first two components were used for plotting, along with the percent variation explained by them. Colors show the temperature specific to each microbial mat, and the shapes correspond to different sampling locations. The strength of statistical significance ($P < 0.05$) for the environmental parameters as explanatory variables is represented by solid arrows.

contribution of minor components, particular the candidate phyla EM3, OD1, OP8, WPS-2, WS4, and WWE1, to the clustering of our samples.

The comparative analysis of beta-diversity was extended to include other hot spring microbial mats described so far, making it possible to obtain the similarity of the microbial mats from this study to others formed in distant geographic areas (Fig. 5).

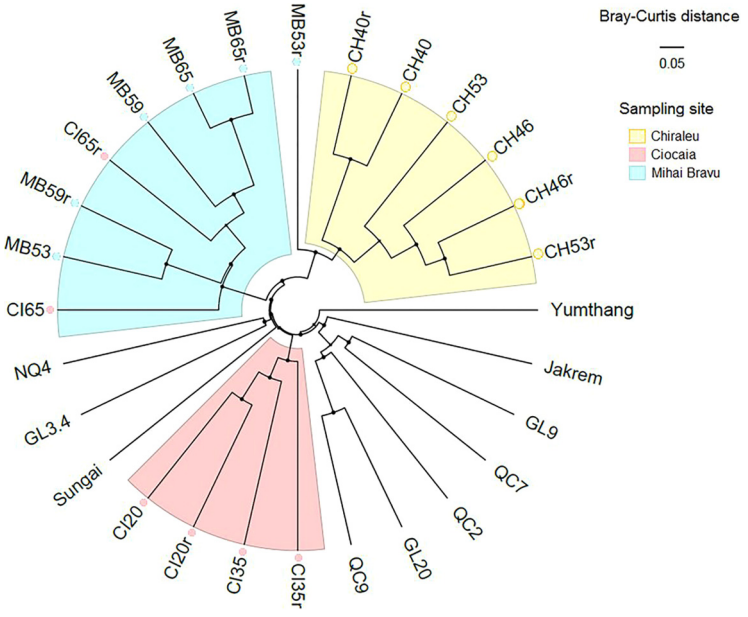

FIG 5 UPGMA tree constructed based on Bray-Curtis distances for a comparative analysis of beta-diversity of the Romanian samples and other hot spring microbial mats reported in the literature. Sample codes correspond to the following locations: Qucai, Tibetan Plateau, China (samples QC9 [SRX206469], QC7 [SRX206468], and QC2 [SRX206467]); Naqu, Tibetan Plateau, China (sample NQ4 [SRX206466]); Gulu, Tibetan Plateau, China (samples GL3.4 [SRX206459], GL9 [SRX206460], and GL20 [SRX206456]) (7); Jakrem, India (SRS932137); Yumthang, India (SRS932073) (89); and Sungai Klah, Malaysia (PRJEB7059) (61).

Once again, the unweighted pair group method using average linkages (UPGMA) tree constructed using the Bray-Curtis distances supported the clustering of microbial mats according to the sampling site, rather than the temperature or the methodological approach (DNA versus cDNA). We can see that samples from CH formed a closed, distinct cluster, probably as a result of being dominated by indicator OTUs included in the *Nostocophycideae*, *Chloroflexi*, and *Betaproteobacteria* groups (Fig. 2; see also Table S2). The CI samples with the lowest temperature (20 and 35°C) are also grouped together, being mainly composed of methanogenic *Archaea*, *Gammaproteobacteria*, and *Oscillatoriophycideae*. The samples at 65°C had increased abundances of *Betaproteobacteria*, and particularly *Hydrogenophilus* members, this being probably the factor that supports their clustering with the MB samples. In this analysis, the Tibetan Plateau spring samples described by Wang et al. (7) formed two clusters based on temperature, as stated in their original research, showing that the temperature is the main driver of the diversity in that area. GL3.4 and NQ4 have similar thermal regimes (48 and 49°C) and are dominated by the phyla *Chloroflexi*, *Cyanobacteria*, and *Deinococcus-Thermus*, while the other samples collected from 60 to 75°C form a distinct group, the majority of OTUs being affiliated with *Chloroflexi* and/or *Aquificeae* (7).

Regarding the similarities among samples, Tobler and Benning (11) also concluded that the differences in thermal regime led to variations in microbial compositions in geochemically diverse Icelandic areas (from 37°C to 45°C). On the other hand, Hjorleifsdottir et al. (24) reported that the observed variability in the community structure of pink-grayish filaments collected from the Hengill area, Iceland, was best explained by the combination of temperature and pH, although geographical distances affected the communities to a lesser extent. As in our case, Purcell et al. (21) found that the microbial mats that developed in three geothermal settings from northern Thailand were clustered according to the sampling sites; they concluded that each site was physicochemically distinct and that the combined influence of temperature and sulfide explained the beta-diversity better than any other combination of environmental variables. All aforementioned studies presented different factors that are shaping the microbial composition and diversity in geothermal areas. Our beta-diversity results add new insights regarding the factors that influence these microbial ecosystems, specifically the Na^+, K^+, HCO_3^-, and PO_4^{3-} ion concentrations that together with temperature may lead to the development of particular microbial mat communities.

Conclusions. The current study characterized unmineralized thermophilic microbial mats from the Western Plain of Romania using a high-throughput metabarcoding approach. For this purpose, partial fragments were sequenced from both the 16S rRNA genes and 16S rRNA transcripts for a more comprehensive discussion of microbial abundance, community composition, and biodiversity. The investigated microbial communities were formed in thermal gradients as a result of uninterrupted water flow from tree drilling holes located in the Ciocaia, Chiraleu, and Mihai Bravu villages. The overall abundances of prokaryotic cells were similar among sampling sites (10^6 to 10^8 copies/mg) and did not show a constant trend along with temperature variation. In all samples, the *Bacteria* domain was far more diverse than *Archaea*, the dominant phyla being *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, and *Nitrospirae*. Methanogenic archaea are most probably metabolically active in the Ciocaia sample developed at 35°C, performing both acetoclastic (e.g., *Methanosaeta*) and hydrogenotrophic (e.g., *Methanothermobacter* and *Methanoculleus*) methanogenesis. As the temperature increases, oxygenic photosynthetic members of *Cyanobacteria* are gradually replaced by anoxygenic phototrophs like *Chloroflexus* and *Ectothiorhodospira*. The functional prediction analysis indicates possible robust biogeochemical cycling taking place in the microbial mats, including carbon, nitrogen, and sulfur cycles, but further functional studies are required to accurately describe the links between specific taxa and particular metabolic roles. The temperature was found to exert a strong control on alpha-diversity, with species richness and evenness proportionally dropping with the increase in temperature. The beta-diversity was found to be influenced to a greater extent by the physicochemical

TABLE 2 Hot springs investigated in this study

Hot spring	GPS[a] location	Sample temp (°C)	DNA sample code	cDNA sample code
Chiraleu	N47.3036 E22.2998	40	CH40	CH40r
		46	CH46	CH46r
		53	CH53	CH53r
Ciocaia	N47.3327 E22.0514	20	CI20	CI20r
		35	CI35	CI35r
		65	CI65	CI65r
Mihai-Bravu	N47.2616 E21.9449	53	MB53	MB53r
		59	MB59	MB59r
		65	MB65	MB65r

[a]GPS, Global Positioning System.

characteristics of each hot spring water, especially by the dominant ion concentrations (Na^+, K^+, HCO_3^-, and PO_4^{3-}), temperature, and electrical conductivity. Although clustering analyses performed in other studies have supported the microbial distribution according to temperature, rather than the sampling location or the geographic distance, our results pointed toward the opposite when describing the patterns of beta-diversity. This hypothesis should be confirmed by extending the current study to include other microbial mats either from Romania or worldwide, from a wider range of temperatures and water geochemistries. Finally, this study provided valuable insights into the biodiversity of these newly investigated hot spring microbial mats, adding knowledge on the factors that might shape the biodiversity of microorganisms in such ecosystems.

MATERIALS AND METHODS

Sampling procedure. Samples were taken in 2013 from microbial mats developed near three abandoned drillings from the Chiraleu (CH; drilling number 4095), Ciocaia (CI; drilling number 4045), and Mihai Bravu (MB; drilling number 605) villages, at temperatures ranging from 20 to 65°C (Table 2). Colorful microbial mats were observed and sampled at each location along the temperature gradient (Fig. 6). In the surroundings of the Chiraleu drilling (Fig. 6A), a green and slimy microbial mat developed at 53°C, without a clear vertical stratification (Fig. 6B), as well as a yellow microbial mat at 46°C which presented a typical laminated structure (Fig. 6C). A thin microbial mat was sampled from 40°C, with a smooth orange surface and green layers underneath (Fig. 6D). Three distinct microbial mats were observed in the proximity of the Ciocaia drilling (Fig. 6E), including green, mucilaginous, and stratified microbial mats forming at 20°C (Fig. 6F), red and slimy microbial mats developing at 35°C that did not show a clear stratification (Fig. 6G), and white microbial mats at 65°C, more consolidated and firm near the substrate and with a mucilaginous, translucent surface layer (Fig. 6H). Microbial mats and streamers were identified near the Mihai Bravu drilling opening (Fig. 6I), including a green, compact, and smooth microbial mat forming at 53°C, with thin layers (Fig. 6J), a stratified microbial mat at 59°C, with an orange top layer and green layers underneath (Fig. 6K), and long, flexible structures forming cream-colored streamers at 65°C along the flowing hot water (Fig. 6L).

The stable characteristics of the water sources were previously reported (91, 92) (Table 1). These three drillings (CH, CI, and MB) tap the Pannonian hyperthermal aquifer, a deposit of neutral to slightly alkaline waters with pH values ranging from 7.75 to 7.91. The high concentrations of Na^+ (676 to 4,120 mg/liter), Cl^- (537 to 1,160 mg/liter), and HCO_3^- (895 to 1,980 mg/liter) ions are probably the result of the static nature of this deposit, which resulted in prolonged water-rock interactions. The collecting rocks of the aquifer were formed under lagoon conditions that allowed the accumulation of dissolvable chloride rocks, which ultimately impact the chemical composition of the thermal deposit (91, 92). The temperatures at the surface and at the bottom of each microbial mat were determined using a field multiparameter (Multi 340i multiparameter; WTW, Weilheim, Germany). As temperatures were similar (±0.1°C) between the top and bottom (data not shown), only the surface values were reported. For DNA and RNA extraction, approximately 3 g of each microbial mat was collected in 15-ml Falcon tubes in triplicate, and TRIzol reagent (Invitrogen) was immediately added to fill up the tubes in order to maintain the integrity of the total RNA. All the samples were transported on ice to the laboratory and processed within the same day. After DNA and RNA extraction, followed by cDNA synthesis, a total of 18 samples were used for prokaryotic cell abundance estimation and metabarcoding (Table 2).

Nucleic acid extraction. Total DNA and RNA were extracted in triplicate for each microbial sample according to the manufacturer's instructions (TRIzol reagent; Invitrogen), with slight modifications. A total of 750 mg of each sample (wet weight) was incubated at 60°C for 10 min, being vortexed prior to and at the end of the incubation time. Then 150 μl of chloroform was added and the tubes were vortexed for 10 s. The tubes were centrifuged at 10,000 × *g* for 10 min. The mixture was separated in three phases: an upper aqueous phase, an interphase, and a lower phenol-chloroform phase. The upper phase was transferred in a new tube for RNA extraction, while the other two phases were used for DNA purification. For DNA extraction, 300 μl of 100% ethanol was added to the lower phases, and the

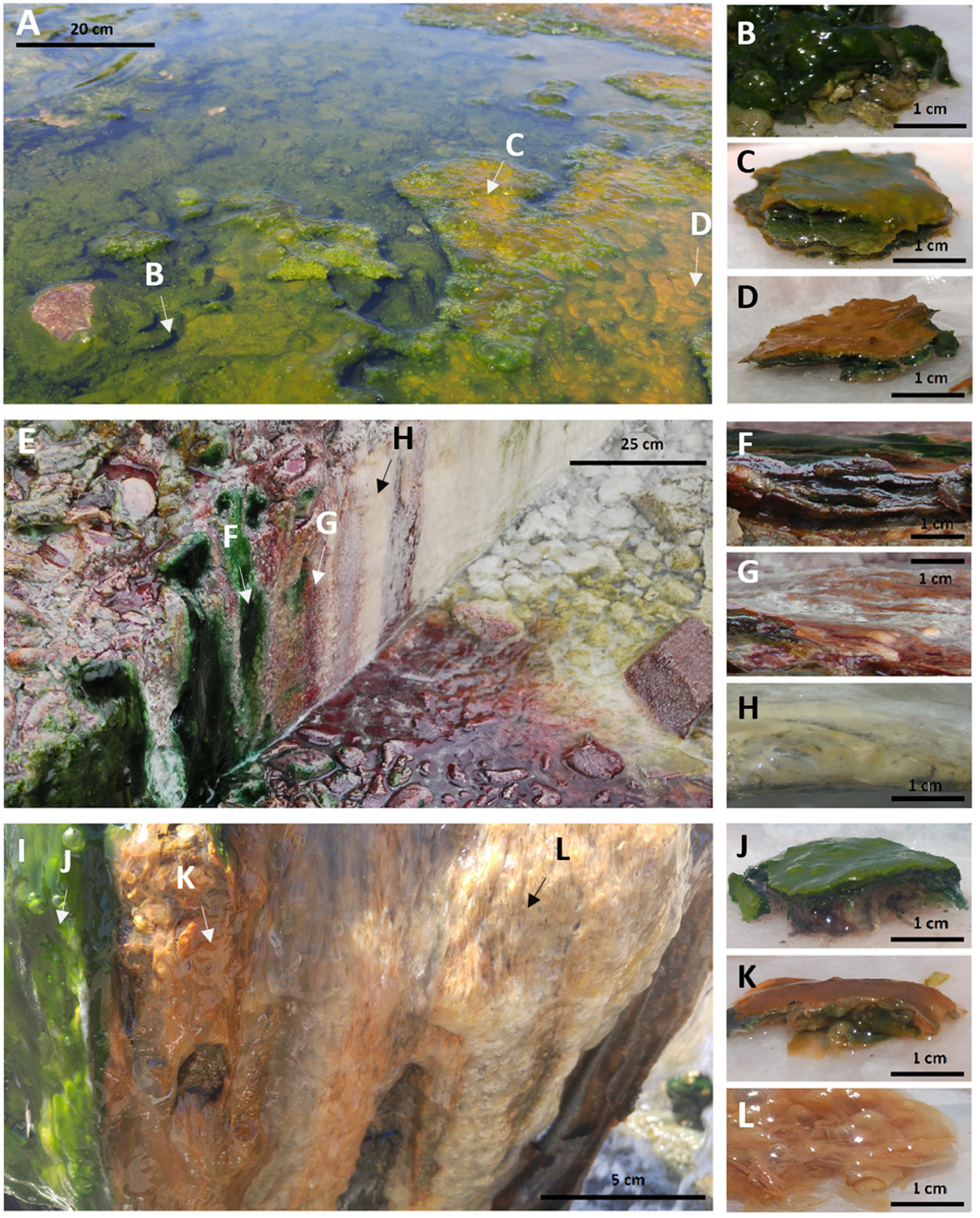

FIG 6 (A) Microbial mats formed in thermal gradients surrounding the Chiraleu drilling (arrows indicate the sampling sites); (B) detailed view of the green, slimy mat developed at 53°C, without a clear vertical stratification; (C) green to yellow microbial mat formed at 46°C, with a typical laminated structure; (D) thin microbial mat developed at 40°C, with a smooth orange top layer followed by green and brown layers; (E) microbial mats in the proximity of the Ciocaia drilling; (F) green, mucilaginous stratified microbial mat developed at 20°C; (G) red and slimy microbial mat formed at 35°C that does not show a clear stratification and which is thinner than the mat at 20°C; (H) white microbial mat developed at 65°C, more consolidated and firm near the substrate and with a mucilaginous, translucent surface layer; (I) microbial mats developed in thermal gradient in proximity of the Mihai Bravu drilling; (J) green, compact, and smooth microbial mat at 53°C, with thin layers; (K) stratified microbial mat at 59°C, with an orange top layer and green layers underneath; (L) long, flexible structures forming cream-colored streamers at 65°C along the flowing hot water.

components were gently mixed by inverting the tubes. Following a 3-min incubation period, the tubes were centrifuged for 15 min at 12,000 × *g* and 4°C to pellet the DNA. The pellet was resuspended in1 ml of 0.1 M sodium citrate in 10% ethanol, pH 8.5, and was incubated at room temperature for 30 min. The tubes were centrifuged for 15 min at 12,000 × *g* at 4°C. This washing step was repeated one more time, and the DNA pellets were resuspended in 1.5 ml of 75% ethanol. The tubes were incubated for another 20 min at room temperature and then were centrifuged for 15 min at 12,000 × *g* and 4°C. The supernatant was discarded and the DNA was air dried for 15 min. The pellet was resuspended in 500 μl of 8 mM NaOH by pipetting up and down. Then the tubes were centrifuged for 10 min at 12,000 × *g* and 4°C, the supernatant was removed, and the DNA pellet was eluted in 35 μl of PCR-grade water. The DNA replicates of the same sample were pooled, and the DNA concentration was determined based on the Qubit dsDNA BR assay kit, using a Qubit fluorometer (data not shown).

RNA extraction and cDNA synthesis. For RNA purification, an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the aqueous supernatant (see previous section) and the tubes were vortexed and centrifuged at 10,000 × *g* for 2 min. The upper phase was carefully transferred to a new tube and mixed with an equal amount of isopropyl alcohol. The samples were incubated at room temperature for 10 min and then centrifuged at 4°C and 10,000 × *g* for 10 min. The supernatant was removed, and 1 ml of 70% ethanol was added to the tubes placed on ice. After 5 min of incubation the tubes were centrifuged at 4°C and 8,000 × *g* for 5 min. The ethanol was discarded and the RNA was resuspended in 30 μl of PCR-grade water. Next, the DNA was removed using the TURBO DNA-free kit (Thermo Fisher Scientific), and the RNA replicates of the same sample were pooled. The RNA concentration was measured using the Qubit fluorometer and the Qubit RNA BR assay kit (data not shown). Total RNA reverse transcription was performed starting with 200 ng of purified RNA for each sample using the First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions.

Quantification of prokaryotic cell abundance by qPCR. The PRK341F/PRK806R primers were used to target the 16S rRNA gene in the total extracted DNA and cDNA by quantitative real-time PCR (qPCR) (93). The reactions were performed in triplicate using the SsoFast Eva Green Supermix (Bio-Rad, Hercules, CA) on a CFX96 Touch real-time PCR detection system (Bio-Rad). The following components were added to the reaction mixture: 7 μl of 1× Sso Fast EvaGreen SuperMix (Bio-Rad), 0.4 μM forward and reverse primers, 20 ng of DNA/RNA, and RNase/DNase-free water to a final volume of 14 μl. The reaction program began with an initial denaturation at 98°C for 120 s, followed by 45 cycles of 10 s at 98°C and an annealing/elongation step of 30 s at 55°C. The gene copy number was calculated by comparing the amplification results to a standard 10-fold serial dilution of known quantities of recombinant plasmids (10^9 to 10^3) carrying the targeted gene. The plasmids were prepared as previously described (94). Additionally, three negative controls were analyzed in the same run. A threshold cycle (C_T) cutoff value corresponding to the lowest C_T reported for the negative controls was used, and the average number of gene copies detected in these controls was subtracted from the sample values.

Preparation of sequencing libraries. The V3-V4 regions of the 16S rRNA gene were amplified from the extracted DNA and cDNA using the PRK341F/PRK806R primers (93) modified by addition of Illumina-specific adaptors. The coverages of the primers against the SILVA reference database were 84.1% for *Bacteria* and 58.5% for *Archaea*. Each PCR mixture (25 μl) contained 1× HOT FIREPol PCR mix (Solis BioDyne, Estonia), 200 nM uniquely tagged forward and reverse primers, 1 μl of sample DNA, and 18 μl of water. The reaction conditions were 95°C for 15 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s, ending with 72°C for 7 min. The amplicon concentration was measured with the QubitdsDNA HS assay kit using the Qubit fluorometer (Thermo Fisher Scientific, USA), and equal amounts of amplicons were pooled for each sample into a normalized library. The pooled library concentration was measured using the PerfeCta NGS quantification kit for Illumina (Quanta BioSciences, USA), and the library was diluted in Tris (pH 8.5) to a final 4 nM concentration. Sequencing was performed on a MiSeq platform (Illumina, USA) using V3 sequencing chemistry with 300-bp paired-end reads.

Amplicon analysis. Raw sequence data were processed and quality filtered through a combination of Usearch v8 and QIIME pipelines (95, 96). Briefly, QIIME was used to extract the barcodes from the sequence data, to join the forward and reverse Illumina reads and to demultiplex the sequence data. Singleton removal and quality control filtering were performed using the Usearch v8 pipeline, by discarding sequences with less than 350 nucleotides and those with more than 0.2 total expected errors (*-fastq_maxee* E option of the fastq_filter command). Remaining quality control filtration included *de novo* and reference chimera removal via the Usearch v8 pipeline, using the latest version of the Greengenes database (13_8) as a reference (97). The resulting operational taxonomic unit (OTU) table was converted into the biological observational matrix (BIOM) format (98). Taxonomy was assigned using the default classifier in QIIME (Greengenes) against the updated 13_8 version of the Greengenes database at a 97% similarity threshold. Next, the mitochondrial and plastidial sequences were filtered out of the OTU table. A multiple-sequence alignment was generated with PyNAST (95) in QIIME, and the alignment was used to build a phylogeny with the FastTree algorithm (99).

Additionally, a comparison of the alpha- and beta-diversity encountered in the Romanian samples to other sequences from hot spring microbial mats deposited in the Sequence Read Archive (SRA) was performed, including the following entries: SRA061437 (7), SRS932137, SRS932073 (89), and PRJEB7059 (61). Because different variable regions of the 16S rRNA gene were amplified and sequenced in these studies, a closed-reference OTU picking was performed in QIIME (95) using a 97% similarity threshold. Prior to this step, sequences were first quality filtered as described in their original studies. The final data set retained only those sequences that match a full-length representative sequence within the last version (13_8) of the Greengenes database.

Statistical analysis. Rarefaction was performed at a depth of 3,499 sequences per sample, followed by alpha- and beta-diversity estimation in QIIME (95). Euclidean distances for physicochemical parameters were computed with the *vegdist* function of the vegan package in R, and the Mantel test (999 permutations) was run to evaluate the environmental impact on the microbial diversity using the weighted Unifrac distances (100). Environmental physicochemical data were log transformed prior to the analysis. PCoA plots were generated for the weighted and unweighted Unifrac matrices, using the *cmdscale* and *envfit* functions of the stats and vegan packages in R. The environmental factors were fitted onto the ordination with the *envfit* function of the vegan package in R, scaled by their correlation to the distance matrix. The significance of the sample grouping according to distinct sampling sites or distinct type of investigated nucleic acids was tested by analysis of similarity (ANOSIM) using 999 permutations (101) in QIIME. Phyla and classes with abundances higher than 1% in the sequencing libraries were plotted using the *inkspot* function of the rioja package in R. The multilevel indicator OTUs (102) for each thermal spring were established using the *multipatt* function of the perm package in R.

Functional prediction. For functionality prediction with PICRUSt (79), the OTUs were picked using an open-reference approach and *de novo* OTUs were removed, keeping in the final OTU-table only the OTUs that had matching Greengenes identifiers (IDs) (13_8). Data in the BIOM OTU table were normalized using the 16S rRNA gene copy number for each OTU, and the normalized table was used for KEGG functional orthologs predictions. In order to evaluate the PICRUSt predictions accuracy for each sample, the weighted nearest sequenced taxon index (NSTI) was computed, an index that describes the extent to which microorganisms from each sample are related to fully annotated genomes; a value of 0.03 meant that on average, microorganisms in the sample were predicted using a relative from the same species.

Accession number(s). The sequence obtained in this study was deposited in the SRA with accession number PRJNA389363.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01363-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This work was partially supported by the EnviroAMR project (grant no. 3499/20.05.2015, financed through the EEA 2009-2014 Financial Mechanism) through the EnviroBioInfo bioinformatic center and by the Core Programme, grant no. PN 16 19 BIODIVERS. C.M.C. and E.S. were also supported by the POSDRU/187/1.5/S/155383 research scholarship program.

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