**Bacterial distribution along a 50°C temperature gradient reveals a parcelled out
hot spring environment**

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Abstract

Understanding the distribution of bacteria is a major goal of microbial ecology which remains to be fully deciphered. In this study, a model 50°C temperature gradient at a Northern Thailand hot spring was analyzed to determine how the bacterial communities were structured in the environment. Communities were examined through 16S rRNA gene amplification, denaturing gradient gel electrophoresis and sequencing. The two major phyla, *Cyanobacteria* and *Chloroflexi*, showed characteristic distributions along the temperature gradient. Different clades were allocated at specific portions of the gradient. Comparisons of the bacterial communities along the temperature gradient showed sharp decreases of similarity at increasing temperature difference. Peaks of maximum richness were observed at 50°C and 70°C. This study contributes to explain how environmental conditions and microbial interactions can influence the distribution of specific bacterial clades and phyla shaping the structure of microbial communities in nature.

14 Introduction

15 Despite the critical role of microorganisms in biogeochemical cycles of elements [50] and the high existing
16 microbial diversity [8], our understanding on the distribution of specific microorganisms in relationship to
17 environmental factors is scarce [8, 36]. The distribution of microorganisms is important to decipher their
18 potential role and biogeography in the environment [24, 33].

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20 Microorganisms have been considered to be able to reach and colonize almost every habitat even those under
21 extreme conditions. This resulted in the hypothesis that ‘everything is everywhere, but the environment
22 selects’ [3, 49] which has had support [24] and opposition [9]. Research is underway to explain the diversity,
23 distribution and biogeographical patterns of microorganisms and if these are in agreement to patterns
24 followed by macroorganisms [9, 24, 36]. In nature, microorganisms thrive under environmental conditions as
25 well as interacting with others. Ecological interactions (e.g., competition, mutualism) among microorganisms
26 are poorly understood although current information suggests that these mechanisms can be of importance to
27 shape the structure of microbial communities [35].

28

29 High temperature environments, such as hot springs, are inhabited exclusively by microorganisms and
30 constitute fairly exclusive environments for allocthonous microorganisms. Besides, these environments show
31 steep temperature gradients within a reduced area allowing the study of the influence of temperature on
32 microbial communities while maintaining other factors under control [26]. Hot springs represent interesting
33 models of microbial ecosystems offering excellent opportunities to investigate fundamental questions on
34 community structure and the distribution of microorganisms [17].

35

36 Most hot springs are characterized by the presence of microbial mats. Numerous reports are available on the
37 communities at Yellowstone National Park [1, 5, 7, 25, 26, 47, 48] and some other hot springs around the
38 world [6, 13, 19, 22, 27, 33, 39]. Thermophilic mats from hot springs have been described to held microbial
39 communities dominated by two phyla, *Cyanobacteria* and *Chloroflexi*. The general believe established along

years of investigation is that these two groups show feedback interactions and share zones with similar temperatures. These groups have been proposed to interact through a *Chloroflexi*'s photoheterotrophic metabolism on low-molecular weight organic compounds produced by the photoautotrophs, the *Cyanobacteria* [48]. In addition, the development of these two groups has been reported to form an exopolysaccharide matrix which constitutes the core of thermophilic mats at hot springs [7, 48]. Recent evidence points towards an opposite perspective because it has been demonstrated that some *Chloroflexi* are able to grow photoautotrophically in both cultures and *in situ* [18, 46] suggesting potential competition [26]. The ecological relationships between *Cyanobacteria* and *Chloroflexi* and their consequences on the structure of thermophilic mat communities requires further investigation.

The aim of this study was to investigate the distribution of the major bacterial phyla and their phylotypes along a wide temperature gradient (up to 50°C). Specific questions to be analyzed were to evaluate how temperature shapes the structure of the microbial community, and the relationships between the major phyla, *Cyanobacteria* and *Chloroflexi*.

Materials and Methods

Sampling site. Samples were collected from a stream initiated from a hot spring at Mae Fang (19° 57.93' N, 099° 09.26' E) (Northern Thailand) (Figure S1). At the source, the hot spring (Figure S2) presented 90°C and water flow showed temperature decreased down to around 40°C. This 50°C temperature range occurred naturally within approximately 100 m. The stability of the temperatures at the hot stream has been confirmed throughout the years. Additional water sources did not exist at the studied portion of the stream. A microbial mat covered the stream bottom (Figure S2) except at the highest temperatures (80°C and 90°C). The geochemical composition of the source water at sampling is presented in supplementary materials on Table S1. Samples were collected from the microbial mats when present or from the sediments at the stream bottom in other cases. Nine duplicate samples were collected at approximately 5-10°C intervals along the stream

(90,0°C; 80,0 °C; 75,0°C; 70,2 °C; 65,0°C; 60,0°C; 55,7 °C; 50,1°C; 39,9°C) and were preserved in RNAlater (Ambion, Austin, USA) on ice until arrival to the laboratory [33]. Herein, results from one of these duplicates are analyzed. Temperature was measured using a digital thermometer and a thermopar microprobe.

Characterization of microbial communities. The major bacterial components of the communities were determined by PCR amplification, a molecular fingerprinting technique, cloning and partial sequencing of the 16S ribosomal RNA (16S rRNA) genes. Previous studies in other Thailand hot springs suggested the existence of a limited diversity in microbial mats from hot waters and the major phylotypes could be easily detected by DGGE analyses [33].

Molecular fingerprinting was carried out using denaturing gradient gel electrophoresis (DGGE) [28] which allowed obtaining a characteristic banding pattern for each community (Figure S3). DNA extraction was carried out using the Nucleospin DNA extraction kit (Mackerey-Nagel, Düren, Germany) [33]. Two completely independent replicates were carried out (Figure S4) and the analysis below was performed on one representative profile from each temperature. Relative quantitative PCR-DGGE analyses have been previously described [31, 33] and this study strictly followed that protocol including the use of migration markers as previously described [34]. The primers used for PCR-DGGE were 341F-GC (5'-CCT ACG GGA GGC AGC AG with a GC-rich tail attached to its 5' end) [28] and 518R (5'-ATT ACC GCG GCT GCT GG) [28].

PCR-DGGE analyses specific for *Cyanobacteria* were performed through a nested PCR amplification. In a first amplification targeting cyanobacterial 16S rRNA genes, the primers cya106F (5'-CGG ACG GGT GAG TAA CGC GTG A) and cya781Ra+b (5'-GAC TAC TGG GGT ATC TAA TCC CAT T + 5'-GAC TAC AGG GGT ATC TAA TCC CTTT) [29] were used. A second amplification from the previous product was carried out as described for bacteria using the primers 341F-GC and 518R.

Phylotypes were differentiated from their migration during DGGE. Different phylotypes result from differences in the banding profiles or fingerprints obtained for a specific bacterial community. Comparisons between DGGE profiles were statistically performed as described by Portillo and Gonzalez [31].

The identification of bacterial phylotypes was carried out by cloning the PCR amplified fragments of 16S rRNA genes. Amplification by PCR of bacterial 16S rRNA genes for cloning and sequencing was performed using the primer pair 27bF (5'-AGA GTT TGA TYM TGG CTC AG) [33] and 907R (5'-CCC CGT CAA TTC ATT TGA GTT T) [20]. For selecting and identifying *Cyanobacteria*, the amplification for cloning and sequencing was the one described above using the primers cya106F and cya781Ra+b. The PCR products were used for the construction of 16S rRNA gene libraries of clones with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). These clones were screened with the protocol described by Gonzalez et al. [10] using PCR-DGGE for the screening of sequences corresponding to the bands in the molecular fingerprints. The selected 16S rRNA clones were sequenced by a third party commercial service (Secugen S.L., Madrid, Spain).

Identification and differentiation of bacteria and their communities. Bacterial taxa were differentiated using the percentage of similarity between sequences. Thresholds at the phylum, 97% and 99% sequence similarity levels for the 16S rRNA genes were selected to group bacteria into OTUs (Operational Taxonomic Unit) [14]. OTUs were defined based on these percentages following sequence similarity groupings [14]. A 97% level of similarity has been approximately related to the species level [37]. Sequences belonging to different bacterial phyla were separated in base to the results from homology search using the Blastn algorithm [2] against DNA databases (GenBank, National Center for Bioinformation, NCBI; <http://www.ncbi.nlm.nih.gov/>). The 16S rRNA gene sequences obtained during this study are available under the accession numbers HQ416721-HQ416869 for those generated using the bacterial primer pair and HQ416870-HQ416899 for the sequences generated using the *Cyanobacteria*-specific primer pair. Rarefaction

curves were constructed according to Hughes and Hellmann [15] based on the OTUs estimated at different levels of similarity.

Different estimates of the variety of microorganisms found in the studied communities were calculated. The Shannon index [12] was computed according to the criteria defining OTUs (see above). Bacterial and cyanobacterial richness were evaluated using the normalized number of different OTUs at each temperature. Phylogenetic distance between OTUs was estimated by the fraction of different nucleotides detected when 16S rRNA gene sequences were aligned and compared. Alignments were performed with the program ClustalW [45]. The microbial communities at different temperatures were compared with the Bray-Curtis similarity index [52]. Mantel tests were performed using the R package *vegan* [30]. Mantel tests were carried out (i) to evaluate the significance of the association between phylogenetic distance and environmental distance determined from *in situ* temperature, and (ii) to determine the significance of differential richness at different temperatures. These tests also indicate the divergence of results from randomness.

Phylogenetic analyses. Aligned sequences (see above) were processed with the program Treepuzzle [42] to obtain tree topologies, using a maximum likelihood quartet puzzling method, showing the phylogenetic relationships between retrieved sequences and their related homologs. Reliability values were calculated for internal branches indicating the number of times (as percentages) a cluster has been reconstructed during the puzzling steps (generally from 5,000 estimations). In order to obtain a conservative tree topology, multifurcations are drawn where the support value for a bifurcation is lower than 50% [41]. The number of nucleotides for tree construction was 760 and 790 nucleotides for *Chloroflexi* and *Cyanobacteria*, respectively. *Thermotoga neapolitana* (Accession Number AB039768) was used as outgroup for tree construction.

Results

The structure of the microbial communities at Mae Fang Hot Springs varied as a function of temperature along the studied gradient. Differences ($p < 0.05$; Portillo and Gonzalez [31]) were observed between the banding patterns of these communities. Specific phylotypes were detected within a few degrees temperature range (generally below 10°C). These results were observed in fingerprints obtained using both bacterial (Figure S3A) and cyanobacterial (Figure S3B) primer pairs.

Rarefaction curves (Figure 1) showed a clear trend towards leveling-off diverging from the 1:1 straight line. The curves obtained at the 97% and the 99% similarity levels were similar suggesting that most differentiation of OTUs occurred below the ‘species’ level (below 97% similarity in 16S rRNA genes). During this study, most phyla populating the studied samples were detected. At 50°C the rarefaction curve did not reach a horizontal asymptote because a richness maximum was observed at this temperature.

A succession of different phyla, from hyperthermophiles to moderate thermophiles and mesophiles, was observed from the highest temperatures to the lowest ones (Figure 2). The hyperthermophiles dominated at the highest temperatures (90°C to 70°C) and these included mainly the phyla *Dictyoglomi*, *Thermotogae*, and *Aquificae*. Lower fractions within these communities were represented by *Thermodesulfovibrio* and the *Deinococcus/Thermus* groups. These hyperthermophiles represented 98%, 81%, 80%, 47%, and 11% of bacterial communities at 90°C, 80°C, 75°C, 70°C, and 65°C, respectively. These hyperthermophilic phyla remained undetected below 65°C. Some uncultivated and barely known phyla, Candidate Divisions, such as the *OP4*, *OP9* and *OP10*, were detected around the 70°C temperature range which suggests a thermophilic adaptation by these candidate phyla. For example, *Armatimonadetes*, previously known as *Candidate Division OP10* [43], was detected as a significant component of the bacterial community at 70°C (13%) and this phylum was present at lower fractions of the communities up to 90°C. This finding suggests that members of the *Armatimonadetes* can inhabit environments at temperatures above those previously reported [32, 41]. The temperature range of 75°C-70°C represented the upper temperatures where *Cyanobacteria* were detected.

Thermophilic bacteria within the phyla *Cyanobacteria*, *Chloroflexi* and *Bacteroidetes* dominated the temperature range from 55°C to 65°C. At 65°C, 39% of the bacterial community belong to the *Cyanobacteria* and this phylum constituted over 20% of the community in the temperature range from 55°C to 70°C. *Chloroflexi* stood for an important fraction of the communities between 40°C to 80°C with a peak at 60°C (46% of the bacterial community). *Cyanobacteria* and *Bacteroidetes* showed similar relative abundances at 60°C and 65°C. *Bacteroidetes* represented 37% of the bacterial community at 65°C and 21% at 40°C. At 50°C, most thermophilic phyla remained at detectable levels and shared the environment with mesophilic and moderate thermophiles. Numerous uncultured phyla were detected at this temperature (50°C) (i.e., *Candidate Divisions AC1, BRC1, KSN1, JS1, OP1, OP3, OP8, KSBI*). Thus, 50°C was the temperature with the highest diversity detected during this study. *Proteobacteria* and *Actinobacteria* were identified at, and below, 55°C. At 40°C, the bacterial community was constituted mainly by the phyla (in decreasing order of abundance) *Chloroflexi*, *Bacteroidetes*, *Firmicutes*, *Chlorobi*, *Cyanobacteria*, *Nitrospirae*, *Proteobacteria*, and *Candidate Division JS1*.

Most bacterial OTUs from the studied hot spring (considering 99% similarity level) were only detected within relatively narrow temperature ranges generally $\leq 10^\circ\text{C}$ (Figures 3). The OTUs expanded at most a few degrees up and down their apparent optimum temperatures. Figure 3 shows the distribution of the major OTUs within the *Chloroflexi* and *Cyanobacteria* along the temperature range under study. The OTUs of these two phyla were classified into seven major groups according to their dominance at a temperature (Figure 3). The *Chloroflexi* of group I were represented by OTUs 25a, 25b, and 26 detected between 75°C and 85°C; *Cyanobacteria* were not detected above 75°C. The group II was dominant at 70°C and was represented by the cyanobacterial OTUs 1a, 1e, 108 and 109 and the *Chloroflexi* OTU 27, dominant in this phylum around 70°C. Group III (around 65°C) was dominated by *Cyanobacteria* and included the cyanobacterial OTUs 112a, 112b, 112c, 115b, and 116. Among the *Chloroflexi* representatives of group III are the OTUs 55 and 56 which showed relatively low representation when compared to *Cyanobacteria* at this temperature. Major OTUs within the *Cyanobacteria* belonging to group IV (60°C) were 115a and 117. Among the *Chloroflexi* group IV

was represented by OTUs 31, 34, and 57. Group V (55°C) was represented by cyanobacterial OTU 110 and the *Chloroflexi* OTUs 29, 30, 32, and 36-37. The *Cyanobacteria* belonging to group VI (50°C) were mainly represented by OTUs 2a and 111, and the *Chloroflexi* by OTU 43 among others at representing minor fractions of the bacterial community at 50°C. The group VII (40°C) corresponded to the mesophilic clades and was represented by *Chloroflexi* OTUs 41, 47, 49, 51, 54 and 59 (among others in lower proportions) and cyanobacterial OTU 113.

Phylogenetic analyses of the two most representative bacterial phyla, *Chloroflexi* and *Cyanobacteria*, detected during this study at Mae Fang Hot Spring, are shown in Figures 4 and 5, respectively. Closely related OTUs inhabiting a specific temperature range often cluster within a phylogenetic branch or closed branches. This suggests a relationship between phylogenetic proximity and similarity of environmental conditions. However, other distantly related OTUs could also be sharing a common optimum temperature suggesting that more than one lineage could be allocated under specific environmental conditions.

The relationship of community similarity versus environmental distance (represented by differences in temperature) was analyzed pairwise using the Bray-Curtis index. The similarity index decreased sharply when increasing the temperature difference between samples (Figure 6). Mantel tests confirmed the significance of this relationship ($p < 0.05$). Generally, beyond a temperature difference of 20°C only minimum similarities were observed.

Comparative estimates of the Shannon index and species richness showed peaks of maximum values at 50°C and 70°C both at 99% similarity OTUs and phyla. A zone with relatively low diversity was observed between 50°C and 70°C where dominated *Cyanobacteria*, *Chloroflexi* and *Bacteroidetes*. The highest phylotype richness was observed at 50°C. The significance of these maximum peaks was tested by analyzing richness of bacterial phylotypes ($p = 0.0034$) and their phyla ($p = 0.0033$) and the Shannon index ($p = 0.0191$ and $p = 0.0045$ at 99% phylum levels, respectively). These richness peaks appeared to represent an edge effect

confluence of the OTUs thriving optimally at temperatures above and below 50°C and 70°C. These two temperatures marked the limits between mesophiles, thermophiles and hyperthermophiles.

Discussion

Analyses of the microbial communities along a 50°C temperature gradient revealed the distribution of different phylotypes within strict temperature ranges limiting their optimal environments. Some phylotypes coexist under specific temperature conditions. Close relatives are more likely to share environmental conditions than distant phylotypes within a phylum which has been shown for the two major phyla in the studied environment, *Cyanobacteria* and *Chloroflexi*. The distribution of bacterial phylotypes along a 50°C temperature gradient shows a parceled out environment with maximum richness at 50°C and 70°C which defines the range of distribution for mesophiles, thermophiles and hyperthermophiles, respectively.

The microorganisms composing the studied communities could be classified into three major groups according to the temperature at sampling: hyperthermophiles (above 70°C), thermophiles (50°C-70°C) and mesophiles (below 50°C). These groups are fairly well defined by temperature conditions and the distribution of the microorganisms detected during this study. Besides, the boundary effects observed at specific temperatures (50°C and 70°C) allow defining ecotones with increased diversity. At the studied hot spring, each one of these three groups inhabits a temperature range, generally, restricted to the limits defined by 50°C and 70°C [16, 40].

A number of uncultivated bacterial groups (i.e., candidate divisions) appeared adapted to thrive within these ecotones suggesting that they require a well defined range of environmental conditions and a potential requirement for metabolic interactions with microorganisms sharing these niches. This might explain current

difficulties to culture representatives of the uncultivated phyla and could present with significant insights for future cultivation attempts.

Interestingly, the distribution of microorganisms (i.e., OTUs) in the studied environment is far from uniform along a reasonable temperature range. Rather, different microorganisms apparently adapted to slightly distinctive conditions are parceled out into relatively narrow temperature-defined zones. Thus, the 50°C temperature range under study is filled up with different microorganisms (i.e., OTUs) out-competing others which develop under slightly different conditions. These observations are well suited to describe the complexity of microbial communities in natural environments and specifically in hot springs.

The temperature ranges available to the OTUs along the gradient are narrower than those usually described for cultured bacteria under laboratory conditions. These findings suggest that microorganisms in nature present different behavior than when isolated and growing under laboratory experimentation. Whilst most OTUs were detected within a <10°C range, cultures often show growth within, at least, a 20-40°C range including an optimum temperature within that range [23, 51]. These findings suggest that the available conditions for the growth of specific microorganisms in a natural environment are fairly restrictive and microbes compete and coexist with other microorganisms within a narrow ecological margin of environmental conditions. Strong competitiveness (and likely strict positive and negative feedback interactions as well) is envisaged in the studied environment due to the tight distribution observed between OTUs along the temperature gradient. These observations constitute a possible explanation for the high diversity detected in natural systems where genetic and phenotypic variability allow for better chances to thrive sharing the environment with other bacteria and under tight environmental conditions.

Results have shown that phylogenic distance is related to environmental conditions. Environmental distance (determined by temperature) suggests phylogenetic differentiation whereas proximity increases the chances of detecting close relatives. Cells are apparently forced to fit in relatively narrow niches. Environmental distance

enhances evolutive pressure towards phylogenetic changes to better adapt to those differential conditions [44]. A taxa-area relationship has been suggested for bacteria [14] as had previously been described for higher organisms [4, 11, 21]. In our study, temperature defines distinctive environments which allow the development of specific microorganisms limiting the growth of others. Thus, specific cells find niches and windows of opportunity at well determined conditions where those OTUs can develop driven by tight interactions with others under similar or slightly different conditions. This packing and sharing of the environmental opportunities structure the complete temperature gradient maximizing the diversity and functionality within the system.

Cyanobacteria and *Chloroflexi* are the two most representative phyla in the microbial mats at Mae Fang Hot Springs within the 50-65°C temperature range. Recent debate has been opened on the co-occurrence [7, 33, 48] or exclusion [18, 26, 46] between these two groups. This study suggests that both hypotheses might explain the distribution and interactions between *Cyanobacteria* and *Chloroflexi* at hot springs. Some of these clades share niches suggesting a potential for metabolic interaction whereas at other temperature ranges apparently one of the phyla is mostly excluded. For instance, in the temperature range 55-60°C *Cyanobacteria* and *Chloroflexi* clades share the environment (Groups IV and V). However, the 65-70°C range is mostly dominated by cyanobacterial clades (Groups II and III). At 75°C and above, *Chloroflexi* clades presented a higher proportion of the community than *Cyanobacteria*. In this case (75°C and above), *Cyanobacteria* exclusion can also be a consequence of temperature inhibition of photosynthesis [1]. Although out of the scope of this study, photosynthesis limitation at or above 75°C does not seem to be a consequence of the photosynthetic machinery as a consequence of measurements of photosynthetic activity at high temperatures [1, 38] and rather it could be attributed to structural damage in these cells (e.g., maintenance of membrane integrity) at those temperatures. The differential distribution of *Chloroflexi* and *Cyanobacteria* clades suggests that potential differences in the physiology of these two bacterial phyla could be governed by temperature. Observed interactions between microorganisms and their environment are not always easy to understand; herein, the presented results suggest that environmental conditions and ecological interactions

(i.e., competition and mutualism) between different clades will ultimately determine the spatial distribution of distinctive phylotypes at hot springs.

Conclusions

A model system such as a broad temperature gradient at a hot spring has been valuable to describe the distribution patterns of microorganisms at the studied system with important consequences for other environments [17]. A variety of different phylotypes tightly packed along the temperature gradient filling the complete environment. The observed diversity was the result of variability within and between clades and phyla. The narrow temperature range where most phylotypes develop indicates strong environmental selection and tight ecological interactions among the members of bacterial communities in the studied hot spring. By expanding the information gathered from a high temperature model environment to other natural environments, these results significantly contribute to partially explain the elevated microbial diversity and complexity existing in nature.

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Figure legends

- Figure 1.** Rarefaction curves for detected phylotypes within the bacterial communities along the studied gradient of temperatures according to their level of similarity (A, 99%; B, 97%; C, phylum). Colours indicate different samples as follows: 80.0°C, marron; 70.2°C, red; 60.1°C, pink; 55,7°C, light blue; 50.2°C, blue; 39.9°C, navy blue. The 1:1 straight line is shown (dashed line).
- Figure 2.** Relative abundance of major bacterial phyla along a 50°C temperature gradient at Mae Fang Hot Spring.
- Figure 3.** Distribution of the major phylotypes within the phyla *Chloroflexi* (A) and *Cyanobacteria* (B and C) along the temperature gradient in Mae Fang Hot Spring. The analyses of *Cyanobacteria* detected during this study by using bacterial domain-wide primers (B) and *Cyanobacteria*-specific primers (C) are shown. Groups based on *in situ* temperature are indicated on top of each figure using roman numerals.
- Figure 4.** Phylogenetic tree showing the clustering of the distinct phylotypes of the Phylum *Chloroflexi* detected in this study and their closest relatives. The temperature groups corresponding to *Chloroflexi* OTUs are indicated. Bar indicates the percentage of substituted nucleotides. Bootstrap values are the result of 1000 iterations. Groups correspond to the following temperatures: Group I, 75°C; Group II, 70°C; Group III, 65°C; Group IV, 60°C; Group V, 55°C; Group VI, 50°C; Group VII, 40°C.
- Figure 5.** Phylogenetic tree showing the clustering of the distinct phylotypes of the Phylum *Cyanobacteria* detected in this study and their closest relatives. The temperature groups corresponding to *Cyanobacteria* OTUs are indicated. Bar indicates the percentage of substituted

nucleotides. Bootstrap values are the result of 1000 iterations. Groups correspond to the following temperatures: Group I, 75°C; Group II, 70°C; Group III, 65°C; Group IV, 60°C; Group V, 55°C; Group VI, 50°C; Group VII, 40°C.

Figure 6. Bray-Curtis similarity index values between bacterial communities along the studied temperature gradient. The comparisons correspond to each of the studied samples collected at 90.0°C (A), 80.0°C (B), 75.0°C (C), 70.2°C (D), 65°C (E), 60.2°C (F), 55.7°C (G), 50.1°C (H), and 39.9°C (I).

Figure 1

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Figure 6