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Low Abundant Bacteria Reflect Soil Specificity—Analysis of Bacterial Communities from Archaeological Investigation of Pre-Industrial Saline Ash Deposits of Bad Dürrenberg (Germany)

Johann Michael Köhler [1,*], Linda Ehrhardt [1], Peter Mike Günther [1], Manfred Böhme [2] and Jialan Cao [1]

[1] Institute for Micro- und Nanotechnologies/Institute for Chemistry and Biotechnology Technical University Ilmenau, PF 10 05 65, D-98684 Ilmenau, Germany; linda.ehrhardt@tu-ilmenau.de (L.E.); mike.guenther@tu-ilmenau.de (P.M.G.); jialan.cao@tu-ilmenau.de (J.C.)
[2] Landesamt für Denkmalpflege und Archäologie Sachsen-Anhalt, D-06020 Halle/Saale, Germany; bat_restoration@yahoo.de
* Correspondence: michael.koehler@tu-ilmenau.de

Abstract: Six soil samples from three layers of an archaeological investigation profile from a pre-industrial ash deposit place have been investigated by NGS analyses of 16 S rRNA. The three pairs of sample originate from top soil (internal reference), from an intermediate ash layer and from a lower ash layer, formed about two centuries ago. In addition to general abundant bacteria, special genera known as halophilic or alkaline-tolerant have been found as expected from the history of the place and from the measured pH-value and conductivity measurements. The close relations between samples of pairs and the differences between the three soil layers are clearly indicated by abundance correlation and PCA-diagrams. Comparative PCA correlation plots including samples from an archaeological excavation site dedicated to pre-industrial coal mining illustrate the high distinguishability of investigated soils. These relations are particular clearly shown when lower abundant bacteria are regarded. The investigations are a further example for the "ecological memory of soil" reflecting the strong human impact on this pre-industrial embossed place.

Keywords: soil; microorganisms; archaeology; human impact; NGS; halophiles; bacterial communities; ashes

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1. Introduction

The compositions of soil bacterial communities are very important for the resilience of ecosystems and for the fertility of agriculturally used areas [1,2]. The use of various agrochemicals in crop production as well as the release of industrial and domestic influences the soil conditions and thus microbial populations. In particular, mining and metallurgy are responsible for massive changes and damage to soils due to acidification and release of toxic metal ions [3–6].

Recent investigations on bacterial communities from archaeological soil samples showed that not only recent land use and modern industrial activities have an impact on soil microorganisms, but also ancient human impacts are reflected by the recent state of soil and the recent composition of soil microbial communities [7–9]. These ancient impacts must not only be considered as early damage to the soil but should also be recognized as contributions to the Beta diversity of soil bacterial communities of a certain area. Variation in the soil bacterial compositions of closely adjacent sampling sites indicates that human activities over many centuries or even millennia have had an impact on the recent state of the soil [10–12]. Due to the smaller scale of land use patterns in most prehistoric, medieval or early modern-period activities, many different soil conditions and microbial communities can be expected in a comparatively small area. Thus, these soils influenced by early human activity could be considered as interesting sampling places for the search of new bacterial strains or consortia with promising tolerance and metabolic traits.

The special effect of early metal mining and metallurgy on soil conditions have become a topic for microbiological investigations since some years, as seen in [5,6,9,13,14]. In contrast, other ancient mining activities such as coal and salt mining have received less attention. In addition to the extraction of coal and salt from the ground, their storage and use should also have left traces in the soil bacterial compositions.

Studies on soil bacterial communities and ecological networks are mainly focused on the higher abundant fractions of OTUs in general. This is very clear for analyzing recent states of microbial activity and ecological interaction. In contrast, lower abundant types including dormant cells contribute to the character and robustness of microbial communities [15] and are—at least partially—related to microbial community and related physiological activities and ecological networks in the past. This is of particular interest for identification of earlier situations in the soil, for the development of soil over longer times and for identification of the consequences of ancient human impacts on the fate of soil at a specific place. In addition to "permanently rare taxa" [16], there are other types of bacteria which are temporary in a state of low abundance, forming the group of so-called "conditionally rare taxa" [17,18], which might show enhanced growth rates when environmental conditions are changed [19]. The main methodological issue of this study was to show that groups of lower abundant OTUs can be involved in the analyses and supply data about the specificity of samples despites the fact that quantitative conclusions related to single, low abundant OTUs are difficult. The utilization of abundant-class separated analysis should be investigated to determine whether higher and lower abundant OTUs convey distinct information regarding sample specificity. The group of lower abundant OTUs should include such "conditionally rare taxa" which are conserved by covering earlier soil surfaces by deposition and translocation of soil material by formerly human activities resulting in top layers covering and protecting formerly top soil layers. On the one hand, the abundance-dependent analysis of relations between bacterial soil communities of different layers in an archaeological profile should give evidence of different environmental conditions in different past periods. On the other hand, the analysis of bacteria from different formerly—but now covered top soil layers— could help for an improved understanding of the dynamics and the ecological function of the "conditionally rare taxa".

The saline activity is an important example of a very long tradition in extraction of a special material. In the region of Halle/S. (Saxony-Anhalt, Germany) intensive saline activities date back to the Bronze and Early Iron Ages, around about three millennia ago [20]. The use of brine from the ground has continued from prehistory to the present day. Here, next generation sequencing (NGS) of 16S rRNA was used for the microbiological characterization of soil samples from an archaeological excavation site where ashes of early industrial saline activities was deposited. This archaeological site is located near Bad Dürrenberg, a famous traditional saline place in South of Halle/S.

2. Experimental

2.1. Soil Samples

The soil samples were collected during an archaeological survey for residues of former pre- and early industrial mining sites and abandoned and devastated facilities in the historical industrial landscape around Halle/S. The samples were taken from a hill locally known as "Ascheberg" (ash hill). The former deposition of ashes was confirmed by an archaeological survey which provided a profile through an old ash deposit site of the Saline of Bad Dürrenberg (southeast of Merseburg; coordinates (GK): R: 4504.487; H: 5685.134; Figure 1). The ash deposit was probably formed in 1836 or little later. The archaeological evaluation of the north side of the hill revealed two ash deposit layers below the top layer (Figure 2). The upper ash layer contained a red-brownish soil material, which obviously formed the surface for less than one year (here called: "short-term ash deposit"). Below this layer, a second ash-containing grey soil layer was identified, which apparently formed the open surface for several years (here called: "long-term ash deposit").

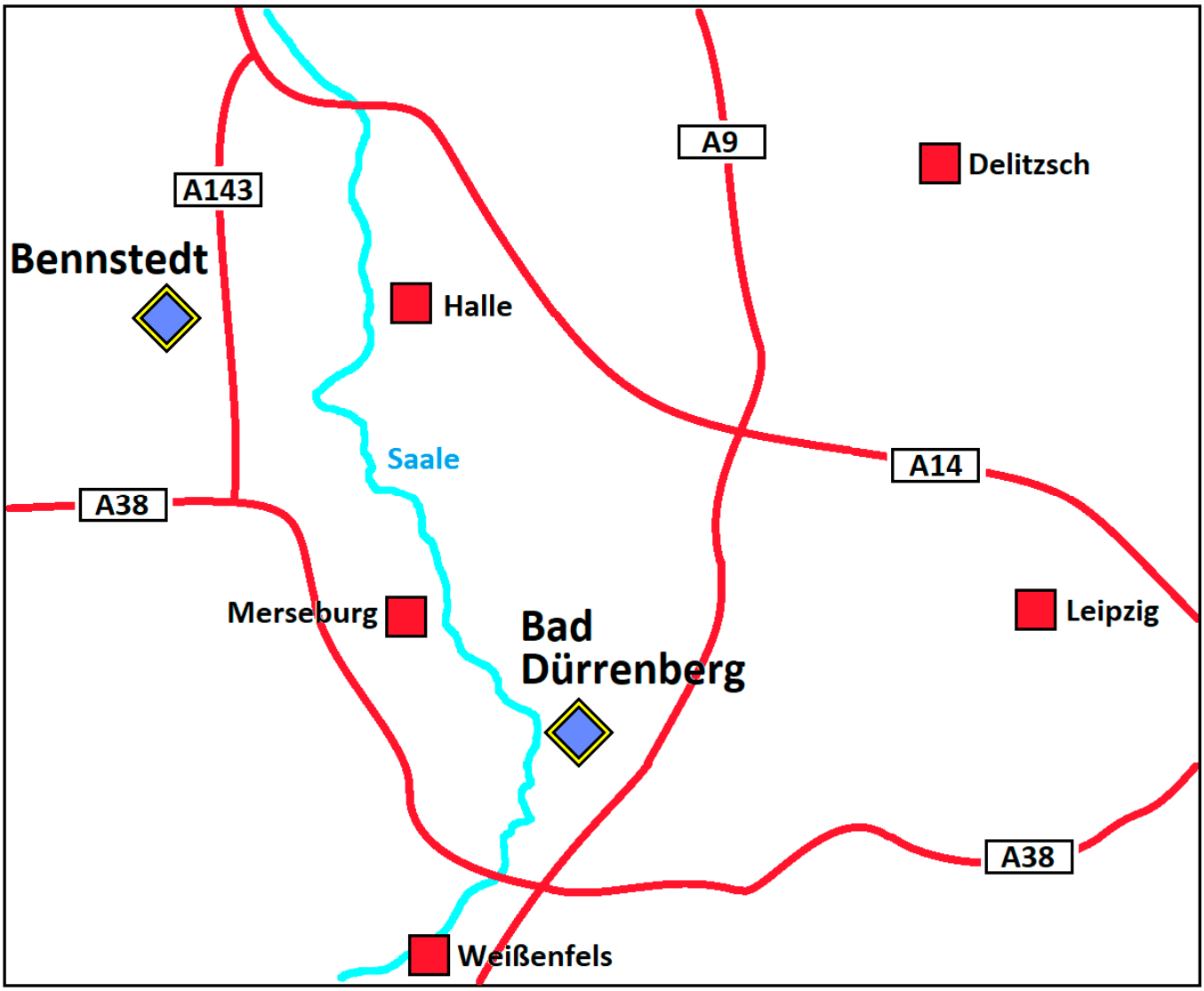

Figure 1. Location of sampling places near Bad Dürrenberg and Bennstedt (blue diamonds: places of sampling).

For the NGS analyses, two soil samples from each ash layer were taken; samples HB61-1 and HB61-2 from the short-term ash deposit, HB62-1 and HB62-2 from the long-term ash deposit. In addition, two samples (HB60-1 and HB60-2) were taken as references from the top soil.

For comparison, also some NGS-data from a sample set of Bennstedt were used, here [12]. These samples had been taken from an ancient coal mine prospection shaft. Their soil properties are clearly different from the soil properties of Bad Dürrenberg (see Table 1).

The soil samples are marked by weak alkaline pH-values, but very high electrical conductivities indicating a remaining high salt content of soil. Obviously, the salt content is particularly high in the deposit layers, but the electrical characterization of the top layer shows a comparatively high salt content too. These analytical data correspond well with the identification of the soil layers as ash deposits and distinguish the soil character considerably from the comparative soil samples of Bennstedt.

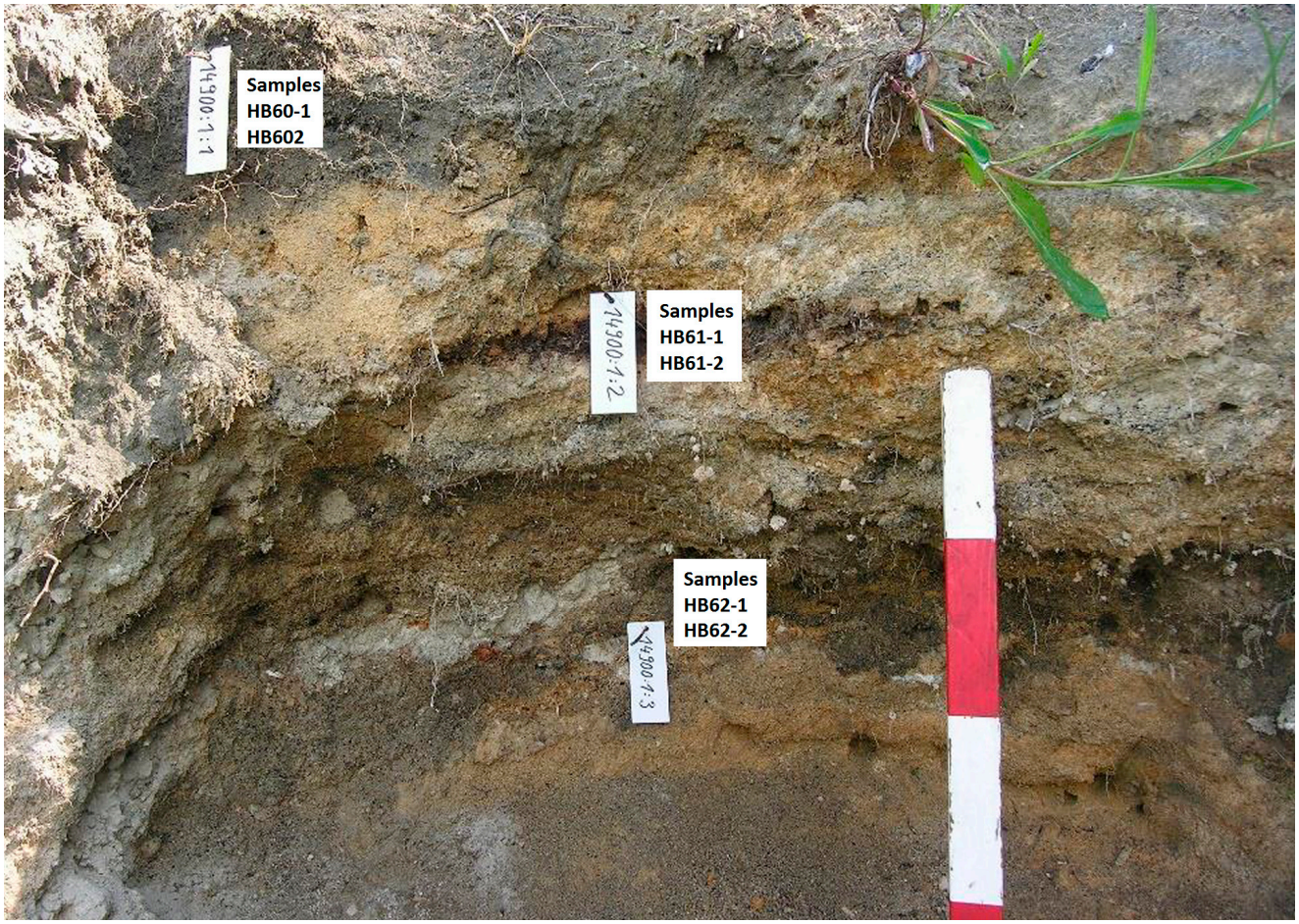

Figure 2. Profile of the historical ash deposit place near Bad Dürrenberg with labelled sampling sites, (photograph taken by M. Böhme during the archaeological investigation).

Table 1. pH and electrical conductivity values of soil samples.

Sample-No	Origin	pH-Value	El. Cond. (µS/cm)
HB60-1	Bad Dürrenberg, top soil	7.7	1068
HB60-2	Bad Dürrenberg, top soil	7.7	1068
HB61-1	Bad Dürrenberg, short–term deposit	8.2	2100
HB61-2	Bad Dürrenberg, short-term deposit	8.2	2100
HB62-1	Bad Dürrenberg, long-term deposit	8.1	2337
HB62-2	Bad Dürrenberg, long-term deposit	8.1	2337
HB57-1	Bennstedt, coal seam	4.2	115
HB57-2	Bennstedt, coal seam	4.2	115
HB58-1	Bennstedt, bright sediment	4.1	43
HB58-2	Bennstedt, bright sediment	4.1	43
HB59-1	Bennstedt, top soil	4.05	45
HB59-2	Bennstedt, top soil	4.05	45

2.2. DNA Extraction, Sequencing and Data Processing

The DNA was extracted directly from the soil samples and the used section of 16S r-RNA was amplified by PCR and the PCR product primarily checked by gel electrophoresis. For a short explanation, a scheme of sample and data processing steps is shown in Figure S2. The details of the whole process of sample preparation, DNA analysis, data processing and taxonomical analysis were performed as described previously [12]. The SILVA database was used for identification of bacteria from the sequence data [21–23].

The data processing (see scheme of process steps in Supplementary Figure S1) resulted in a table that provides the abundances of phyla and lower taxonomical types for each investigated sample. The taxonomical identification of bacteria by the 16S r-RNA analyses allowed in many cases a resolution down to the genus level. In other cases, only higher taxonomical levels were identified. The finest possible identification level led to bacterial types called "Operational Taxonomical Unit" (OTU), which are genera in the optimal case. Many of the found OTUs are related to bacterial types in the on-line database, but they were marked as "uncultured". These bacteria and some others are known from their genetic characteristics but were not cultivated and further characterized until now.

The PCAs were performed using the logarithm-related r-values:

$$r = \log_{10}(1 + N/N_{sum})$$

where N is the abundance of single OTU in a sample and N_{sum} the total number of all reads of a sample.

In addition to the PCA, hierarchical cluster analyses were performed in order to check the relations between the different samples and sampling sites. For these analyses, the r-values (log-related abundance values) were applied too. For clustering and displaying the results in dendrogramma, the related functions of Matlab have been used. The clustering was performed for different abundance classes in order to distinguish the different suitability of higher and lower abundant OTUs for characterization of specificities and similarities between the samples and sampling sites.

3. Results and Discussions

3.1. Composition of Soil Bacterial Communities on the Level of Phyla and Selected Classes, Orders and Families

The composition by phyla clearly shows the similarity within the sample pairs, but also certain differences between the sampling sites (Figure 3). The top soil samples HB60-1 and HB60-2 are marked by the lowest content of *Proteobacteria*, but a comparatively high content of *Archaea* and *Chloroflexi*. Soils HB61-1 and HB61-2 (short-term ash) soil showed a high read number of Firmicutes when compared to the other both sampling sites. The long-term ash soil (samples HB62-1 and HB62-2) are marked by the highest content of *Acidobacteria* and the lowest content of *Actinobacteria.*

The differences between the samples are more pronounced when the bacterial types are compared at lower taxonomic levels. However, this comparison initially confirms the similarities between the single samples in the sample pairs. In the domain of *Archaea* (Supplementary Materials, Figure S3), the top soils (HB60-1 and HB60-2) are marked by comparatively high contents of *Nitrosophaeraceae* and *Cand. Nitrocosmicus*. The HB61 samples differ from the other both sampling sites by a comparatively low content of *Cand. Nitrososphaera*. Both the ash deposit sampling sites (HB61 and HB62) are distinguished from the top soil (HB60) by a comparatively high number of reads for the group SCGC AAA011-D5 belonging to the order *Woesearchaeales* (phylum *Nanoarchaeota*).

The phylum *Acidobacteriota* is mainly represented by *Blastocatellia* (Supplementary Materials, Figure S2). In addition, *Holophagae* and *Acidobacteriae* are mostly abundant in the top soil (HB60) but less in HB62 (long term ash deposit). The short-term ash deposit is marked by the highest portion of reads for *Vicinamibacteria.* Significant differences between the sampling sites were also observed within the class *Blastocatellia* (Supplementary Materials, Figure S4): the top soil showed significant reads for the groups DS-0100, 11-24, *Blastocatella* and *Aridibacter*, which are missing in the ash deposit soils. The long-term ash deposit has the lowest diversity within the class *Blastocatellia*. This class is represented there only by uncultivated *Blastocatellaceae*.

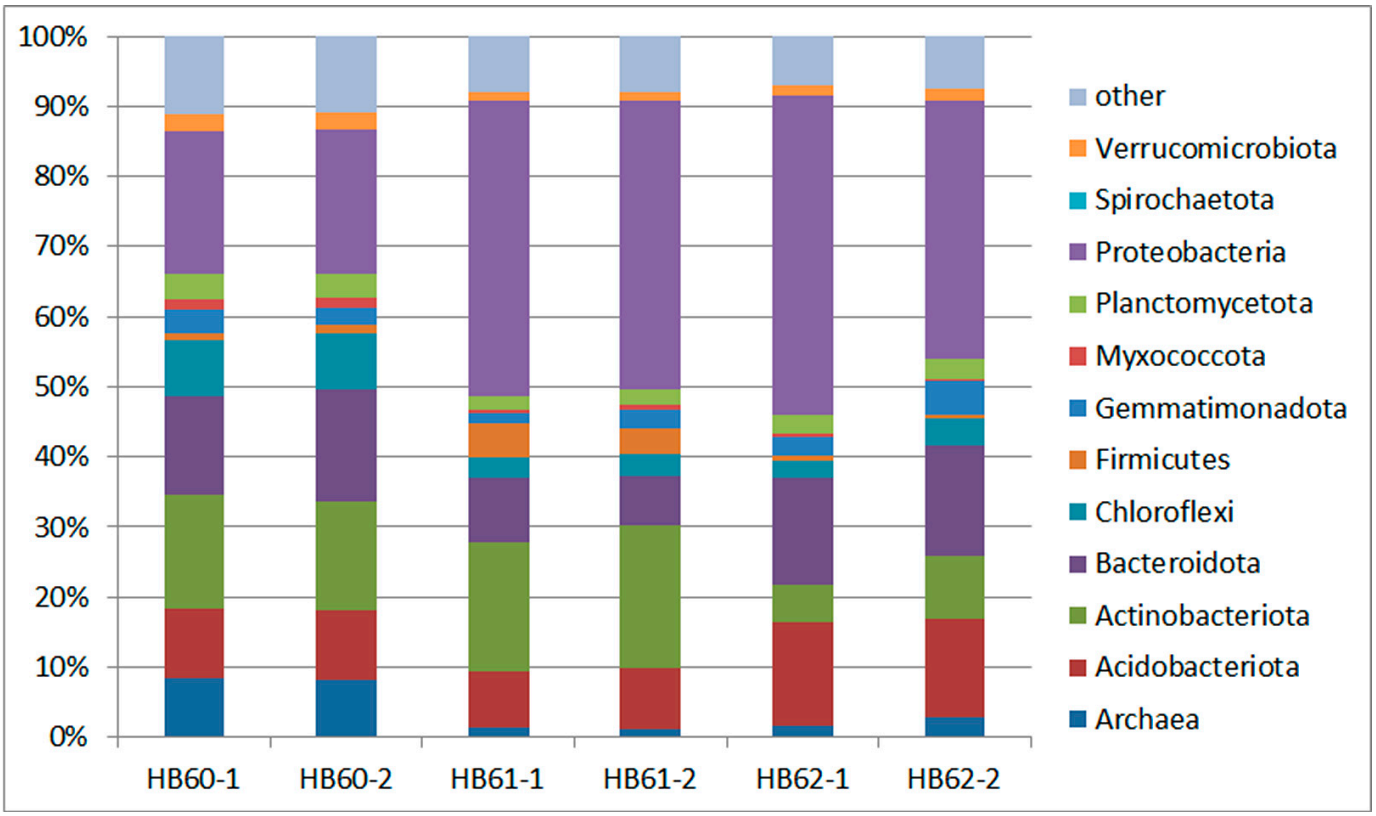

Figure 3. Composition of soil bacterial communities from the archaeological profile of the pre-industrial saline ash deposit of Bad Dürrenberg by most important phyla.

Mostly quantitative differences appeared between the sample pairs in the phylum *Actinobacteriota* (Figure S5). The top soil (HB60) is marked by a particular high portion of *Gaiellales,* the short-term deposit (HB61) by the highest contents of *Actinomariales* and *Nitriliruptorales,* the long-term deposit (HB62) by the highest content of *Micromonosporales.*

Significant diversity and differences between sampling sites reflect the order *Rhizobiales* (Supplementary Materials, Figure S6) and the family *Sphingomonadaceae* (Supplementary Materials, Figure S7, both *Alphaproteobacteria*), too.

3.2. Comparison of Soil Bacterial Communities on the Level of Operation Taxonomical Units (OTUs)

3.2.1. General Correlation of OTU Abundances

The general quantitative relations between pairs of samples are well illustrated by logarithmic correlation plots showing the similarity relations for all observed OTUs. Each point in these diagrams represents one OTU. The correlation diagrams for OTUs in sample pairs confirm the high similarity in the abundances of the single OTUs inside the pairs from the same sampling site (Figure 4a–c). All highly abundant OTUs of one top soil sample are also highly abundant in the other top soil sample (Figure 4a), and the same was observed for short-term ash layer (Figure 4b). Very few exceptions were observed for the long-term ash layer (Figure 4c). But the high correlation confirms the high reproducibility in the analytical procedure. It is important to note that there was a correlation observed, also for mediate and—to some extent,—for lower abundant OTUs. This suggests that even OTUs with only a few reads can provide information about relations between samples when considered in groups.

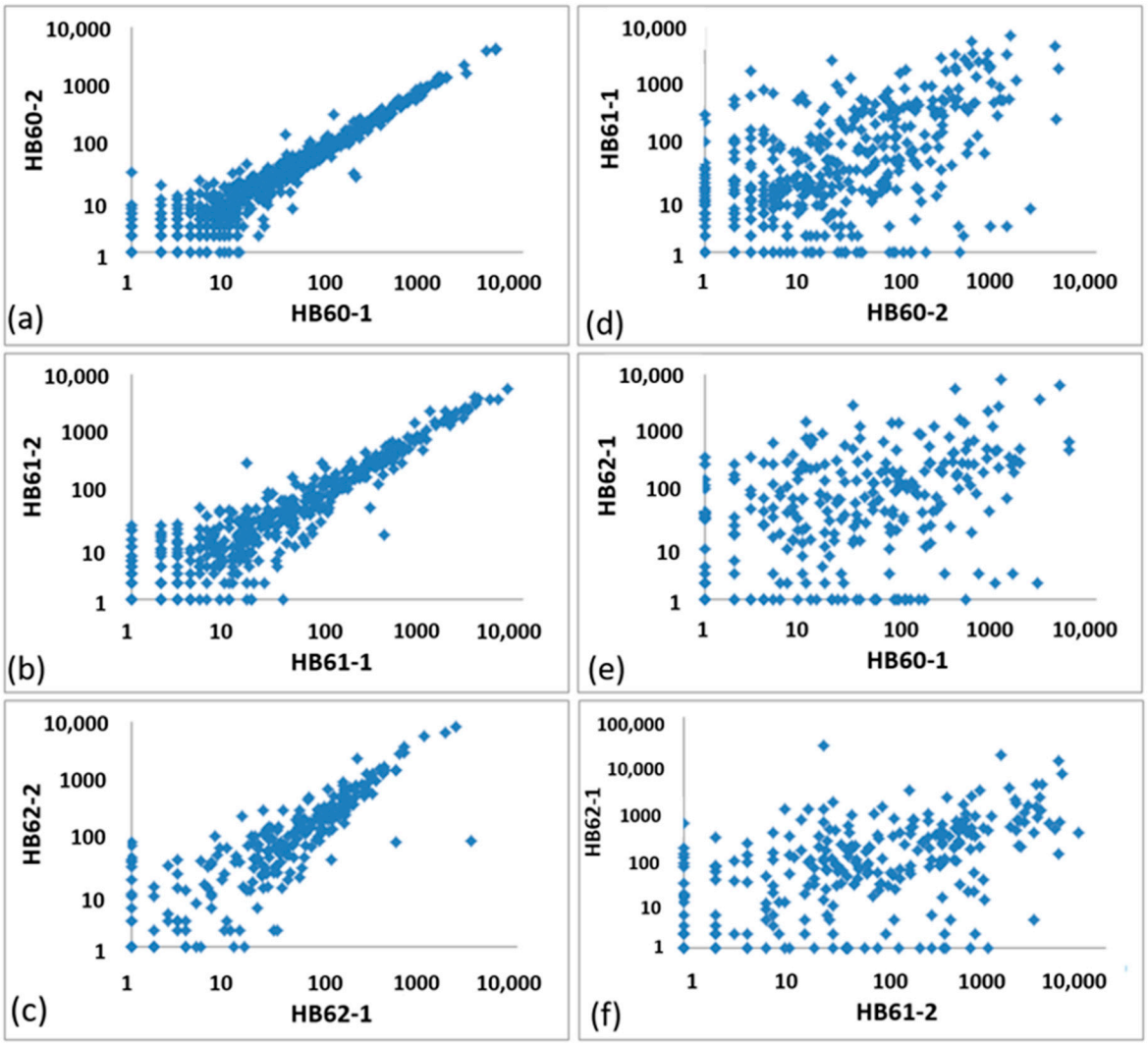

Figure 4. Double-logarithmic correlation plots for pairs of samples by abundances for single OTUs present in both samples of the related pair. (**a**–**c**) Correlations between bacterial communities in the pair samples. (**a**) Top layer, (**b**) short-term ash layer, (**c**) long-term ash layer. (**d**–**f**) Examples of sample pairs of different archaeological layers. (**d**) Comparison between short-term ash layer and top layer, (**e**) comparison between long-term ash layer and top layer, (**f**) comparison between long-term and short-term ash layer.

A completely different picture is observed when looking at the correlations between OTUs from different samples. The diagrams comparing sample pairs of different layers reflect strong differences between their soil bacterial communities, as shown for three examples (Figure 4d–f). A weak correlation was found only for higher abundant OTUs above about 100 reads in total. The comparison between the long-term ash layer and the top soil showed the lowest correlation (Figure 4e), whereas relatively higher correlations are found between the pairs of neighborhood layers as top soil and short-term ash (Figure 4d) and short-term and long-term ash (Figure 4f).

3.2.2. Dominant OTUs

This general difference between the communities in the three investigated layers was also found when the dominant OTUs are compared (Figure 5). Uncultivated members of the families *Microoscillaceae* and *Xanthobacteraceae* are the only similarity if the five most abundant types are regarded. Some top abundant OTUs are different; for example, *Bacteriap25* and members of *Nitrosophaeracea* in top soil (Figure 5a), *Bacillus* and uncultivated

Actinomarinales in short-term ash layer (Figure 5b), uncultivated members of *Blastocatellaceae* and *Pseudomonas* in the long-term ash layer (Figure 5c).

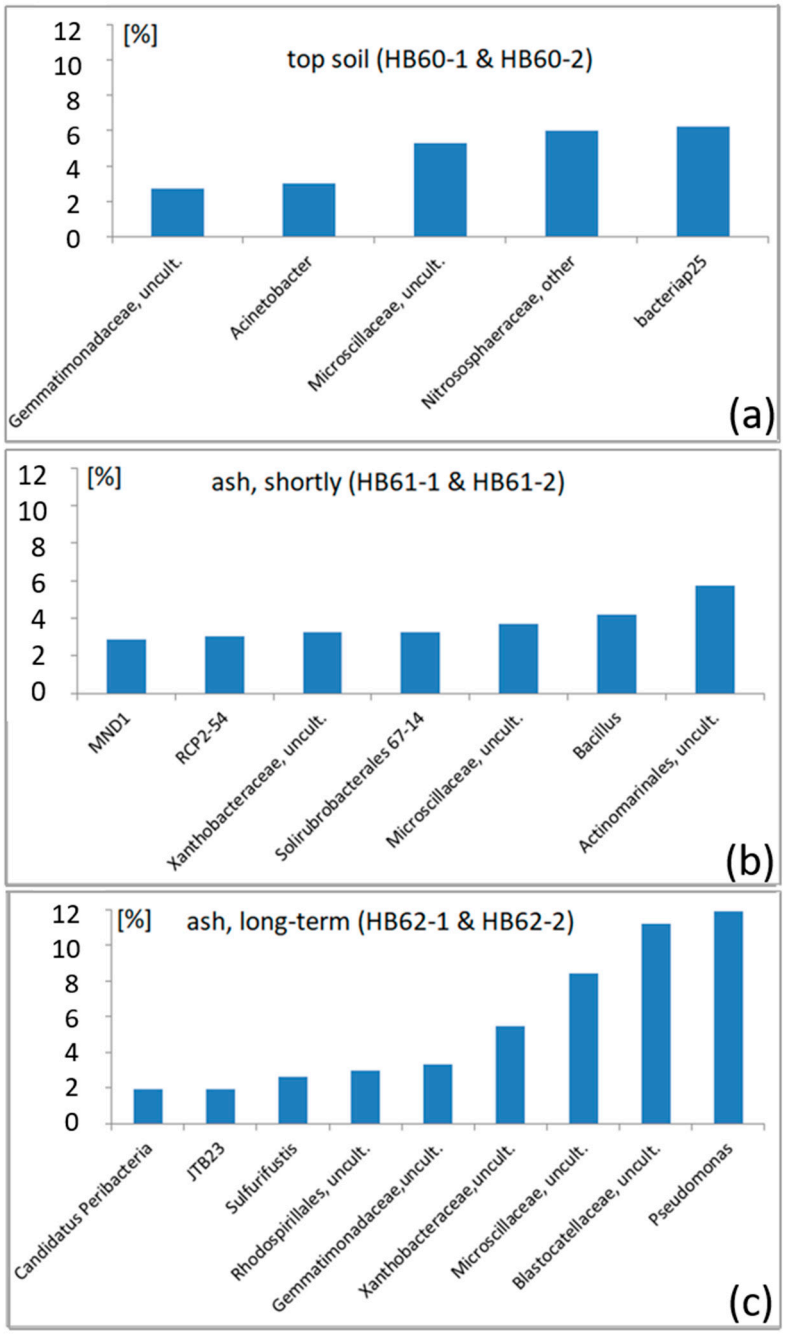

Figure 5. Percentages of reads of most abundant OTUs in the three layers: (**a**) top soil; (**b**) short-term ash, (**c**) long-term ash.

In the top soil (HB60-1, HB60-2), *Acinetobacter* is among the most abundant genera, too (Figure 5a). These bacteria are highly abundant in soils in general and are known for their ability to decompose organic substances, including aromatic compounds such as benzoic acid, hydroxyl benzoic acid and tryptophan, but also toxic chemicals such as phenol, halogenated aromatics and components of mineral oil. Therefore, *Acinetobacter* species are not only of interest for bioremediation applications but can also be interpreted as indicators of the local presence of the mentioned contaminants.

Bacillus and *Pseudomonas*—found as two of the most abundant genera in both the ash layers—belong to the most abundant soil bacteria overall. Interestingly, in addition to others, a high number of reads were found for *Sulfurifustis* in the long-term ash deposit (HB62-1, HB62-2). It represents about 2% of the total number of reads in these samples (Figure 6b). *Sulfurifustis* is a thermotolerant genus and is known to be able to oxidize sulfur and some of its compounds [24].

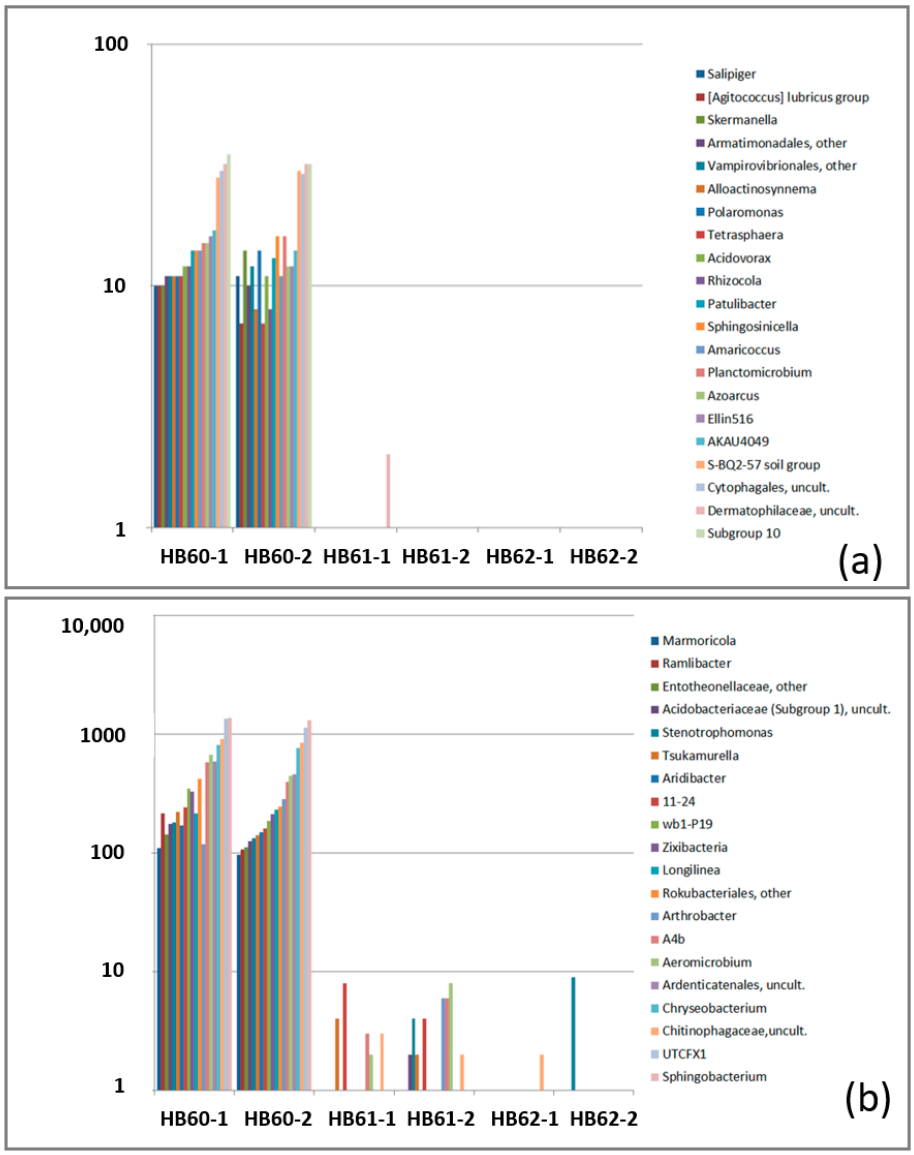

Figure 6. Group of OTUs which are preferably or exclusively found in the top soil samples (numbers of reads). (**a**) OTUs exclusively or nearly exclusively present on sampling point HB60; (**b**) set of OTUs preferably found on sampling point HB60 (top soil Bad Dürrenberg).

In addition to *Acinetobacter* (HB60), *Pseudomonas* (HB62) and *Bacillus* (HB61), the three sampling sites are marked by some dominant bacteria that have not yet been cultivated (Figure 5). All three sample pairs are clearly distinguished from each other by the most abundant OTUs. This may be due to the specific soil conditions of the three different sampling sites on the ash deposit place.

3.2.3. Sample-Specific Types

Each of the sampling sites is marked by a characteristic group of bacteria that is absent or very low in abundance at the other sampling sites. Around 40 OTUs have been proved by

NGS reads from both the top soil samples (HB60-1 and HB60-2), where they are exclusively or preferentially present (Figure 6a,b). Some halophilic types are also found in top soil, such as the genus *Salipiger*, which was first described from hypersaline habitat in south-eastern Spain [25]. The appearance of specific halophilic bacteria in the top soil is not surprising. It results from the fact that the underground and the environment are affected for least two centuries by the saline activities including the sole processing as well as the burning of coal and the deposition of ashes. A high content of salt in the top soil is indicated by the comparatively high value of electrical conductivity (Table 1), although the fact the conductivity of the ash layers is even higher.

Other OTUs are preferentially or exclusively found in the ash soil samples (HB61 and HB62). Some of them are found in all ash soil samples at higher abundances than in the top soil, including *Chitinophaga*, *Leptospirillum*, *Jiangella*, *Pseudolabrys*, *Haloactinopolyspora*, *Arsenicitalea*, *Promicromonospora*, *Sporichthya*, *Methyloceanibacter* and *Immundisolibacter* (Figure 7a,b).

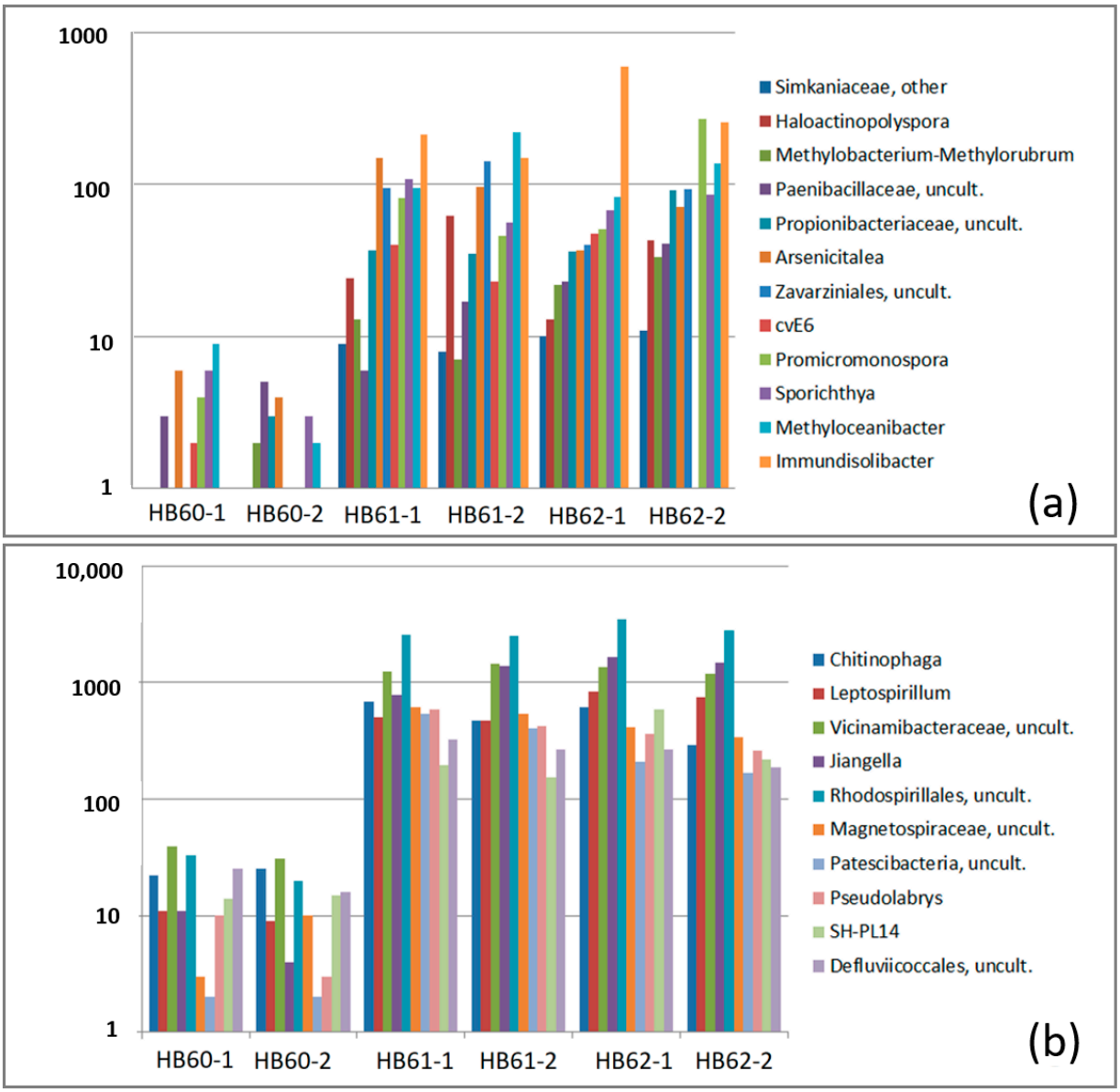

Figure 7. Abundances of OTUs which are preferably or exclusively found in the samples from the both ash layers: (**a**) OTUs with less than 10 reads in the top layer samples, (**b**) OTUs with more than 100 reads in all ash layer samples.

The presence of halophilic bacteria is also reflected by this group of bacteria, which is preferentially found in the ash layers. *Haloactinopolyspora* was identified in a Chinese salt lake. The highly halophilic organism was found to grow in solutions containing up to 23% NaCl [26]. The moderate thermophilic genus *Methyloceanibacter* was found in a marine sediment near to a hydrothermal vent. It belongs to the nature of marine microorganisms that they are salt-tolerant overall [27].

In addition to the salt, the ash deposition also caused a concentration of other contaminations on the ash hill and its environment. Probably, this includes arsenic, indicated by *Arsenicitalea*, a highly arsenic-tolerant bacterium which was first found in sediment containing high arsenic concentrations [28]. The following are mostly found either in one of the ash deposits only: *Glacihabitans*, *Microcella*, *Tepidamorphus*, *Raoultella*, *Micavirbrio*, *Kazania*, *Rhodobacter*, *Caulobacter Aliihoeflea* and others show reads exclusively in the short-term ash deposit samples HB61-1 and HB61-2 (Figure 8a). *Pseudofulviminoas*, *Pelagibacterium*, *Rhodopirellula*, *Egicoccus*, *Nitriliruptor*, *Novosphingobium* and *Variibacter* have preferentially been found there (Figure 8b). Some of these bacteria are known from saltwater environments or alkaline conditions, and what obviously confirms this is that the bacterial communities of the ash layers had adapted to the enhanced salt content in the soil as well as to the moderate alkalinity. *Microcella* was firstly described from a highly alkaline groundwater sample [29]. *Aliihoeflea* was isolated from tidal flat sediment [30]. The halophilic *Pelagibacterium* was firstly isolated from the China Sea [31]. The alkaliphilic bacterium *Nitriliruptor* was found in a soda lake sediment. It grows up to 2 M NaCl [32]. *Egicoccus* is an alkaliphilic and halophilic bacterium, too. It was first isolated from a saline–alkaline soil in northwestern China [33].

Edaphobaculum, Cand. Azambacteria, Nitrosarchaeum, Lacunisphaera, Polycyclovorans, Achromobacter, Taibaiella, Ferrovibrio, Rhizorhapis and Bordetella belong to the OTUs found with the highest abundance in the samples of the long-term ash deposits HB62-1 and HB62-2 (Figure 9a,b). Some of these bacteria have special metabolic features. Nitrosarchaeum is an ammonia-oxidizing mesophilic aerobic organism [34]. A strain of Ferrovibrio was isolated from sediment of a low-salinity spring. It is capable of reducing nitrate by using Fe(II) as electron donator [35]. Polycyclovorans is a strictly aerobic and halotolerant bacterium. It was isolated from a marine diatom [36]. It is able to metabolize both aliphatic and aromatic hydrocarbons. The presence of Polycyclovorans in all three layers and their particularly high abundance in the long-term ash deposit may indicate hydrocarbon contaminations of the ash hill due to the use of coal and the burning processes during the salt production.

3.3. General Sample Specificity of Low Abundant OTUs Shown by Comparative Principle Component Analyses (PCA) and Comparison with Soil Bacterial Communities from an Archaeological Excavation Site near Bennstedt (Germany)

A general comparison with samples can be obtained by rank diagrams of OTU abundances. These rank orders are shown in logarithmic graphs in Figure 10. It can clearly be seen that the top soil contains the highest number of OTUs and is marked by a smooth rank function (Figure 10a,b). In contrast to that, the short-term ash shows a small shoulder at mediate abundant OTUs (around No 400–500, corresponding to abundances between about 100 and 1000 (Figure 10c,d)). The lowest number of OTUs and a strong shoulder for lower abundant OTUs was observed in case of long-term ashes (Figure 10e,f). It is assumed that this shoulder indicates a disturbance of bacteria distribution by ancient human impact resulting in a rank function reflecting that the numerical distribution of OTUs is not so balanced as in case of top soil with the smooth rank function.

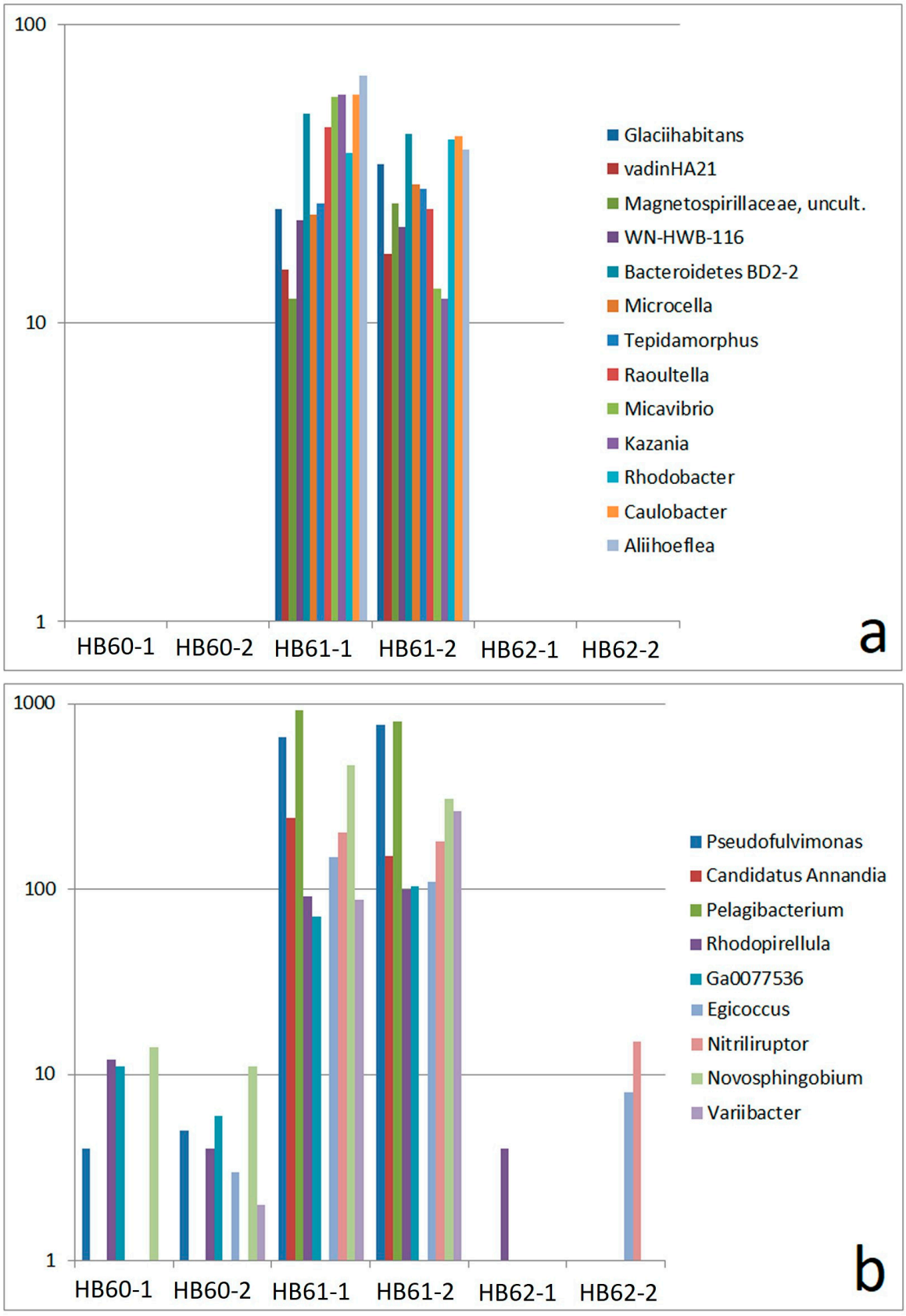

Figure 8. Group of OTUs which are exclusively (**a**) or preferably (**b**) found in the short-term ash layer (numbers of reads).

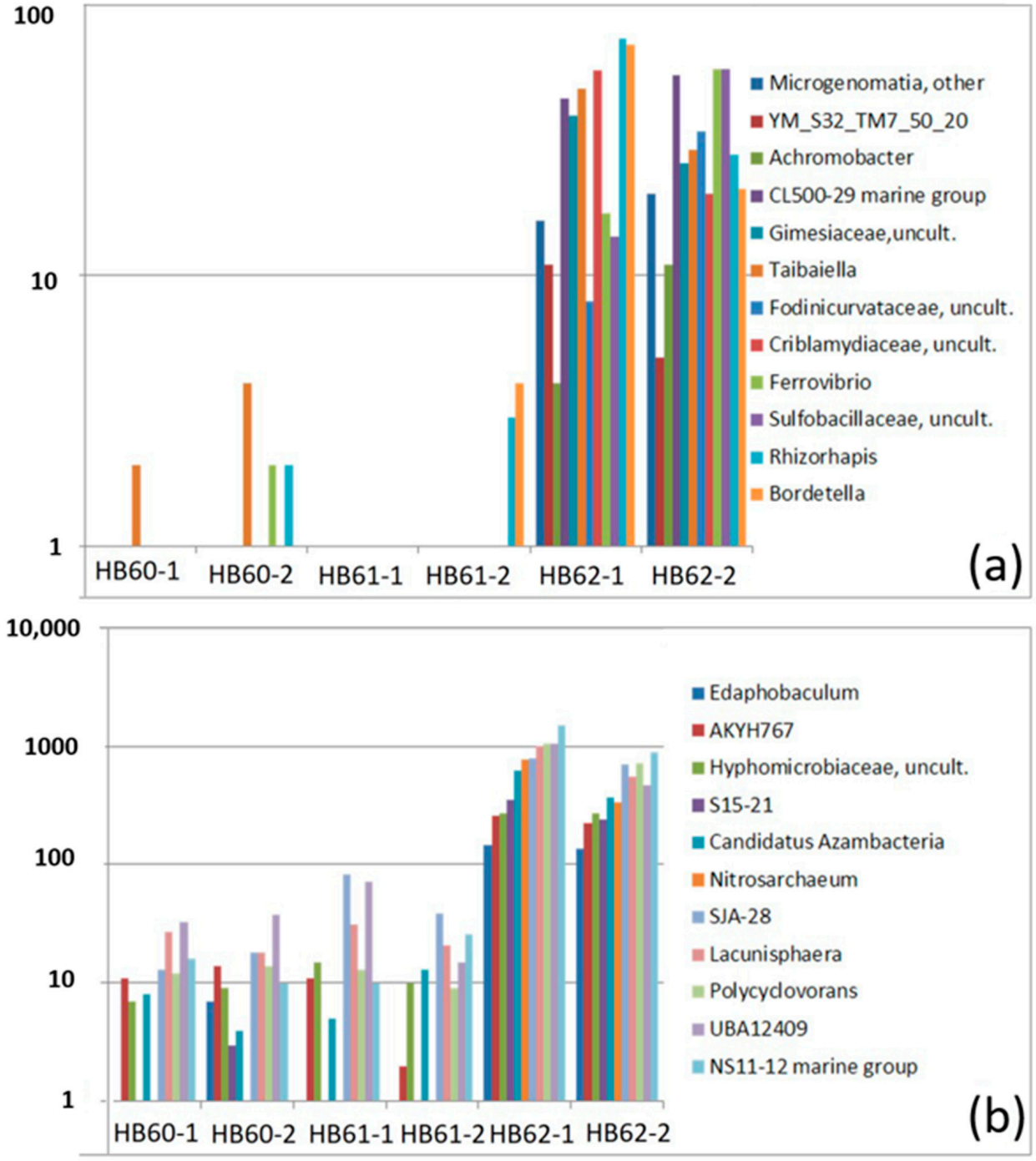

Figure 9. Abundances of OTUs which are nearly exclusive (**a**) or preferably (**b**) present in the samples from the long-term ash layer (numbers of reads).

A similar strong shoulder was observed in the rank diagram of samples from the translocated bright sediment found inside the shaft hole of the pre-industrial coal prospection near Bennstedt (samples HB58-1 and HB58-2). There was already proposed to interpret such a shoulder as a disturbance of smooth numerical distribution of abundances in soil bacterial communities which might be due to the previous transfer of soil material from one environment to another [12]. Conversely, such an interpretation would suggest that the presence of moderate to lower abundant OTUs, or their DNA, retains information about the ecological history of a location.

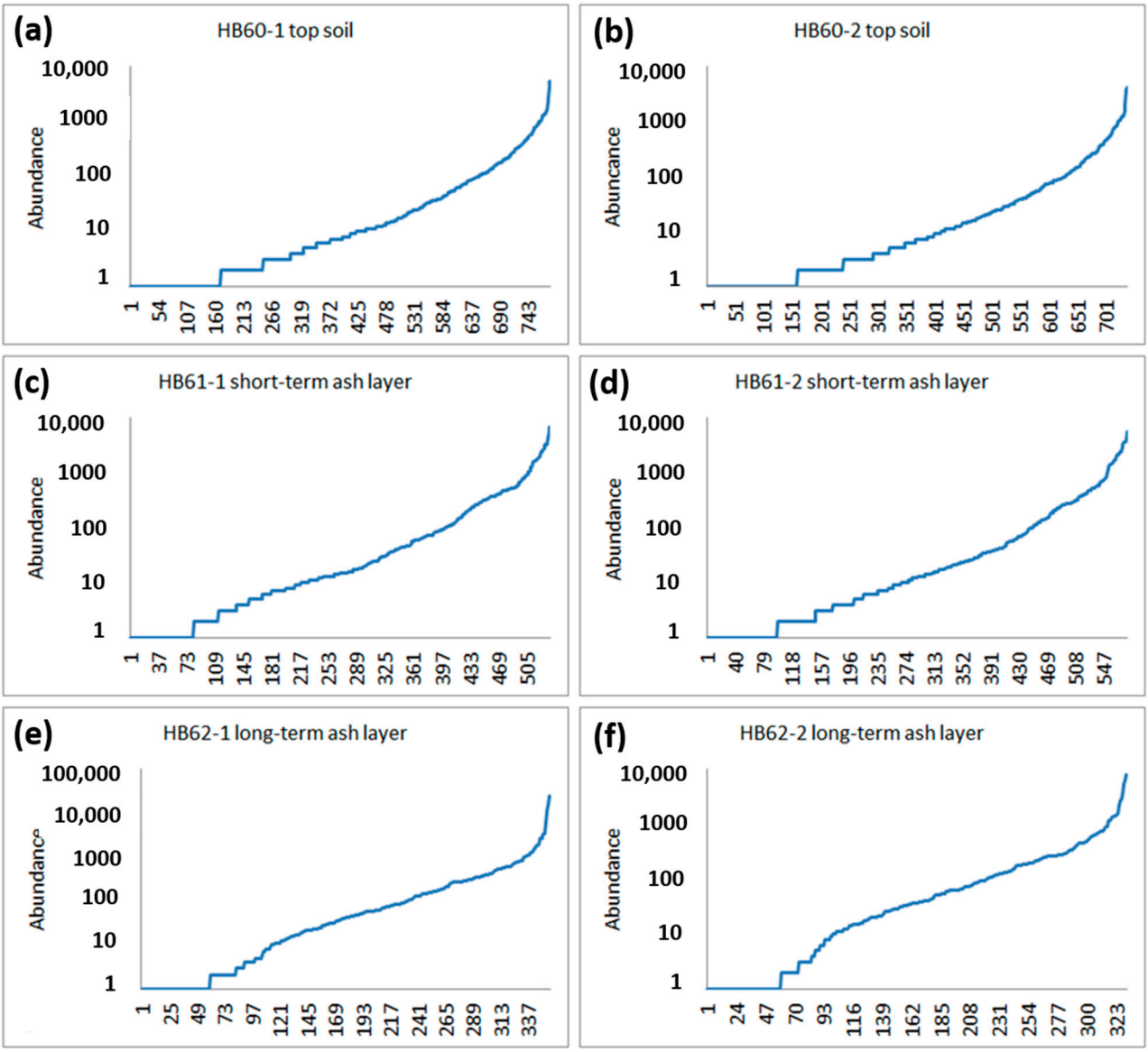

Figure 10. Rank order graphs for all OTUs of the six samples from bad Dürrenberg: (**a**,**b**) top soil samples; (**c**,**d**) samples of the short-term ash layer; (**e**,**f**) samples of the long-term ash layer. (Abundances (=read numbers) in dependence of rank numbers of single OTUs.).

For the evaluation of the results, the data from the saline samples from Bad Dürrenberg were compared with the data of the soil samples from the ancient coal mine exploration shaft near Bennstedt [12] also by PCA. The results of earlier studies on soil samples from human impact areas suggested the high importance of lower abundance bacterial types for distinguishing the character of local soil bacterial communities [37]. Therefore, here, the data of the saline samples of Bad Dürrenberg were analyzed together here with the samples from Bennstedt by a PCA distinguishing the abundance classes of single OTUs. Therefore, the total abundance of all regarded samples with a total of ten or more reads in total (i.e., the sum of all considered samples) is divided into the following four abundance classes: (a) more than 10,000 reads, (b) between 1000 and 10,000 reads, (c) between 100 and 1000 reads and (d) between 10 and 100 reads.

The result for the four abundance classes is shown by PC1/PC2 correlation plots in Figure 11. For the highest abundances (>10,000 reads), two clusters can be clearly distinguished concerning the samples from Bad Dürrenberg on the one hand and the samples from Bennstedt, on the other hand (Figure 11a). Both places are very strongly separated for

the first principle component. This picture is not surprising and can be explained by the spatial nearness of the sampling sites of both locations and by the fact that the pH-values of the samples from Bad Dürrenberg are in the range of 7.7–8.2 (slightly alkaline), whereas the samples of Bennstedt showed a low pH (around 4.1; acidic). The single samples of samples pairs taken from the same sampling spot are not well distinguishable in the group of most abundant OTUs.

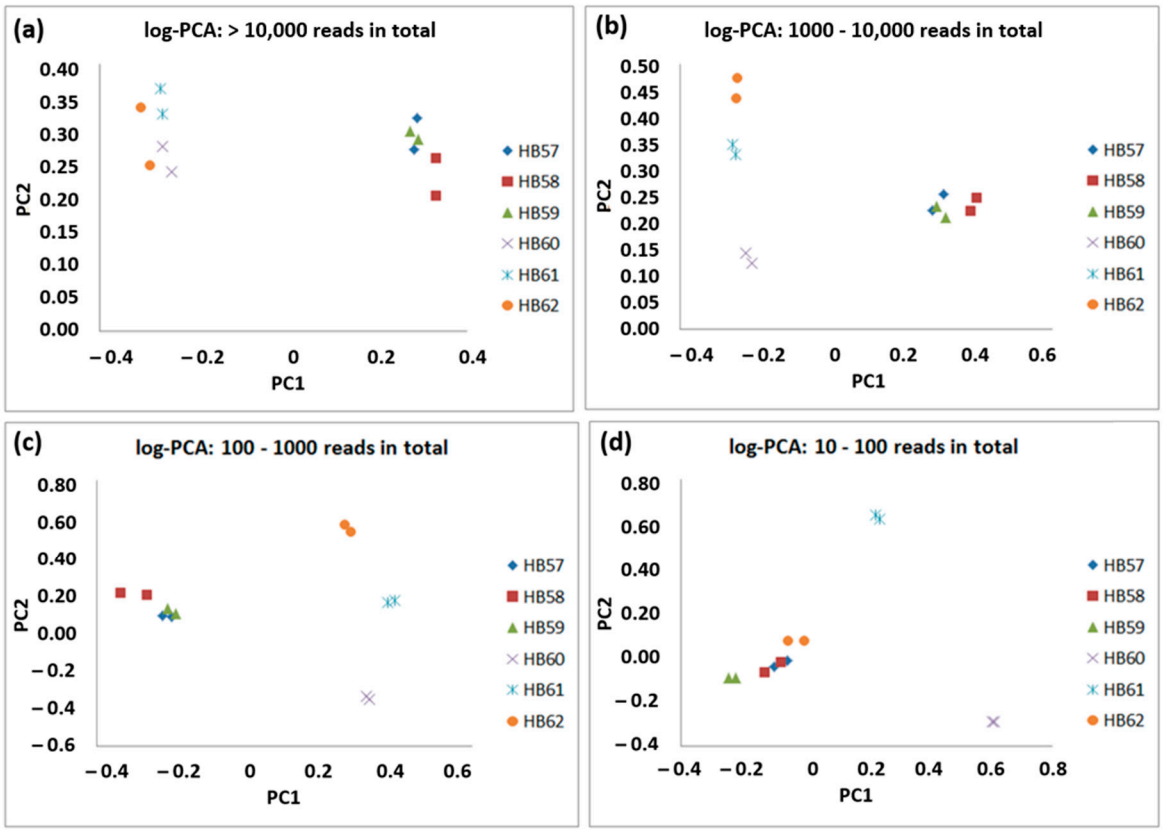

Figure 11. Diagrams of the correlations between first and second principal component of the comparative Principle Component Analyses of samples from Bad Dürrenberg and samples from Bennstedt [12] for different total abundance classes (sums of abundances in all 12 regarded samples). (**a**) For OTUs with more than 10,000 reads, (**b**) for OTUs with 1000–10,000 reads, (**c**) for OTUs with 100–1000 reads, (**d**) for OTUs with 10–100 reads.

This situation changes in the middle and lower abundance groups. Between 100 and 10,000 reads, the samples of Bennstedt still form a closed group that differs from the samples from Bad Dürrenberg, but the three sample pairs from these places are clearly distinguishable from each other by the second PC (Figure 11b,c).

Astonishingly, both the long-term ash samples (Bad Dürrenberg, HB62) are found close to the cluster of samples from Bennstedt in the group of the lowest abundant OTUs (Figure 11d) despite the distant location and the strong differences in pH. It seems that in the context of all samples other criteria than pH and salt content are responsible for the composition of the lower abundant section of the soil bacterial community.

In contrast, the other both sample pairs from Bad Dürrenberg, the short-term ash and top soil, confirm their special and different character, but also the high similarity within the pairs, even at the lowest abundances. For the group of lower abundance OTUs, the samples from the long-term ash layer, on the one site, and the samples from the short-term ash layer are in large distance for the first and the second principal component (blue stars and violet stars in Figure 11d).

The abundance-class-dependent analysis of data by hierarchical clustering confirm the high similarity between the samples in the sample pairs in contrast to the differences between the sampling sites (Figure 12). But, still more than the PCA, the dendrograms from hierarchical clustering support the assumption that the high abundant bacteria reflect preferably the recent ecological situation, whereas lower abundant OTUs are—at least partially—related to formerly ecological situations in the past and reflect more specific characters of soil bacterial communities and of the ancient situation.

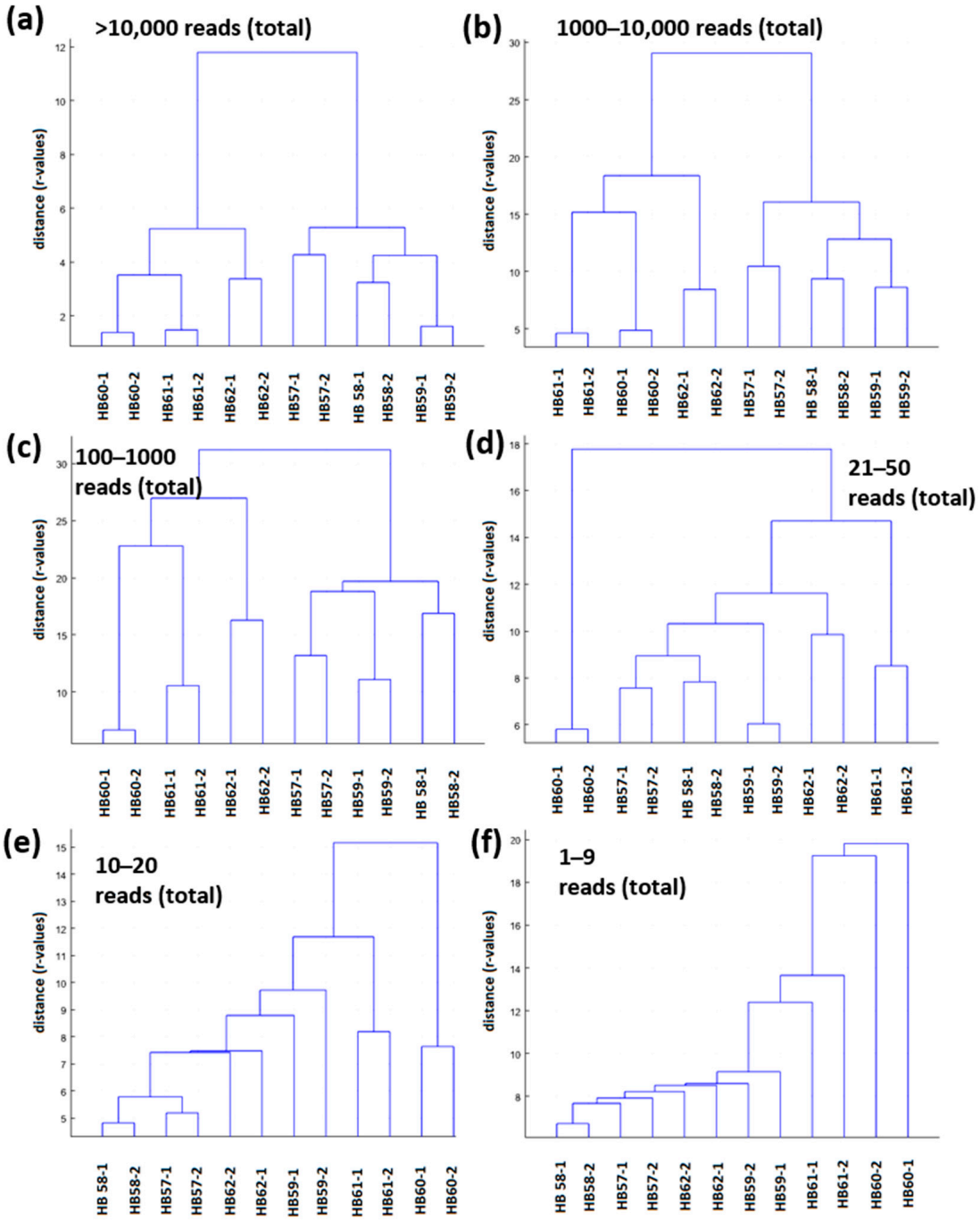

Figure 12. Results of abundance class cluster analysis for investigating similarities and differences between sampling points and investigated places: (**a**) dendrogram for OTUs with more than 10,000 reads in total, (**b**) dendrogram for OTUs with 1000 to 10,000 reads in total, (**c**) dendrogram for OTUs with 100 to 1999 reads in total, (**d**) dendrogram for OTUs with 21 to 50 reads in total, (**e**) dendrogram for OTUs with10 to 20 reads in total, (**f**) dendrogram for OTUs with 1 to 9 reads, only.

The differences in the soil bacterial communities between the ash deposit place of Bad Dürrenberg (HB60, KB61 and HB62) and the ancient coal prospection place near Bennstedt (HB57, HB58 and HB59) are very clearly shown in the clustering of the OTUs with more than 10,000 read in total (sum over all 12 samples) as displayed in Figure 12a. This distinguishability is also verified for abundances between 1000 and 10,000 (Figure 12b) and between 100 and 1000 (Figure 12c). For still lower total abundances, the specific differences between the single sampling sites and both the investigated places get higher importance (Figure 12d). But even for the class of total abundances between 20 and 50, all sample pairs of the six sampling sites shows the highest similarity with each other. The high similarity in sample pairs from the same sampling site is also confirmed for four sample pairs (HB57, HB58, HB60, HB61) in the group of OTUs with total abundances between 10 and 20, only (Figure 12e). Only, at very few read numbers (1–9 in total), the differences between single samples becomes more evident than the differences between sample pairs and investigated places. For lower abundances, the top soil samples (HB60) and the upper ash deposit samples of Bad Dürrenberg (HB61) show the highest differences to all other samples.

The hierarchical clustering can also be applied for classification of OTUs. Here, we analyzed the 20 OTUs with the highest read number in total. The resulting dendrogram is displayed in Supplementary Materials, Figure S8.

4. Conclusions

The three investigated layers of the archaeological profile of the ash deposit can be clearly distinguished by bacterial groups with large differences in their abundances in the layers. These bacterial groups form characteristic abundance patterns. A significant proportion of the layer-specific bacteria as well as bacteria preferentially appearing in one of the investigated layers are known as to be halophilic or alkaliphilic. Others appear to be related in the metabolism of organic contaminants.

The comparison of the soil bacterial communities reflected in the NGS-data of Bad Dürrenberg and the formerly described archaeological excavation site of Bennstedt shows important differences. They can be partially explained by large differences in soil pH and salt content, which are reflected in the measured electrical conductivities. However, these differences between the samples from Bad Dürrenberg and Bennstedt are not related to the large differences in salt content and pH, exclusively. The comparatively high similarity between the long-term ash samples with all Bennstedt samples for the group of low abundant bacteria (10–100 reads in total across all samples) suggests an additional relation, which seems to be independent of pH and electrical conductivity of the salt. The components of the bacterial community that are linked to the previously deposited layer of saline and are also found in the coal mining area can be speculated on. It could indicate a functional relation or be related to use of coal from mines of the environment and the displacement of unburned soil material. It seems that a considerable part of OTUs with lower abundances has to be regarded as "conditionally rare taxa" [17,18], which are related to environmental conditions in the past and are related to the historical human impact on soil microbial communities and the relocation and conservation of soil material by deposition of top layers.

In addition to the direct comparison of abundances of phyla and lower taxonomical levels down to the genus levels or OTUs, relations between samples can be characterized by PCA of the abundance data. The PCA of all investigated samples show a particularly high distinguishability between soil layers and places for OTUs with moderate and low abundances.

The results confirm the high comparability in the pairs of samples on the one hand and the significant differences between the sampling sites on the other hand. The results clearly reflect the similarity inside the sampling group from Bad Dürrenberg on the one side and the sampling group of Bennstedt on the other side if highly and moderately abundant

OTUs are considered. In contrast, the cluster analysis of lower abundant OTUs shows individual differences between the single sampling points and the single samples.

The investigation result of the three soil layers from the ash hill from Bad Dürrenberg correspond well to the expected situation due to the deposition of pre-industrial ashes about two centuries ago. The results confirm that microbiological investigations of soil bacterial communities can provide site-specific characteristics of places with ancient human impact and can help to understand how formerly human activities have affected for the local ecological situation to date.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments11030042/s1. Figure S1: scheme of process steps from archaeological excavation to data analysis. Figure S2: relative abundances of selected *Archaea* in the samples from Bad Dürrenberg. Figure S3: relative abundances of dominating families/orders of *Actinobateriota.* Figure S4: relative abundances of selected OTUs inside the class *Blastocatellia*. Figure S5: relative abundances of most abundant orders in the phylum *Actinobacteriota.* Figure S6: relative abundances of families in the order *Rhizobiales*. Figure S7: relative abundances of OTUs in the family *Sphingomonadaceae*. Figure S8: result of hierarchical clustering (dendrogram) applied for all 12 sampling places and related to the 20 most abundant OTUs in total. The left-hand branch of the tree (a) contains the OTUs which are most frequent in the samples of Bennstedt (low soil pH). The right-hand branch (b) relates to the most abundant bacteria in the samples from Bad Dürrenberg.

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