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Article

NGS Data of Local Soil Bacterial Communities Reflecting the Ditch Profil of a Neolithic Rampart from Hachum (Germany)

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Featured Application

The presented investigation demonstrates the applicability of Next Generation Sequencing data of soil bacterial DNA for differentiation of soil regions of an archaeological profile of a neolithic rampart Ditch. The obtained sequence data from the central interior part of an archaeological investigated Ditch of Hachum (Germany) can be used for clearly distinguishing the related soil bacterial communities from reference sampling points. The differences have been reflected by quantitative comparison on the level of phyla, by correlation diagrams and by comparing the distribution of special operational taxonomical units.

Abstract

An archaeological exposure near Hachum, featuring a Ditch profile interpreted as part of a Neolithic earthwork, was characterized using DNA analyses of bacterial 16S rRNA from soil samples. The results showed that the middle and lower parts of the Ditch fill could be clearly distinguished from each other and from the surrounding area based on the composition of soil bacterial DNA. Genera detected predominantly in the lower part of the Ditch suggest that, after the Ditch was completed, organic matter, animal dung, and possibly even human feces were accumulated at the bottom. The investigations demonstrate that analyses of soil bacterial communities can provide valuable insights into the history and function of a Neolithic earthwork and, more generally, represent an important additional source of information for interpreting archaeological contexts that are devoid of or poor in finds.

Keywords: DNA sequencing; archaeology; soil; bacteria; neolithikum; rampart; Ditch profile; cattlebreeding

1. Introduction

The powerful technologies for the sequence analysis of minute quantities of DNA have not only become essential tools in medicine, biotechnology, and forensics, but are also opening up new insights for archaeology. The decoding of human gene from bones and the determination of ancestry and kinship relationships of individuals from the Stone or Bronze Age, as well as the clarification of prehistoric migration processes, are important examples of the significance of DNA analyses for reconstructing societal developments in prehistoric, non-literate times [1–3].

While the analysis of human remains and pathogens using DNA has been employed for some time and has led to important findings, comparatively little attention has so far been given to the analysis of environmental microorganisms and their potential for investigating archaeological sites [4–6]. However, individual studies have shown that soil microorganisms present a kind of ecological memory [7–12] and can also provide clues to past human activities. The soil microbiome and its DNA

can be seen as a storage of information on past human influences on the soil and can thus provide insights into such activities [13–19].

Changes and specific compositions of soil bacteria are reflected in archaeological samples from buried soil layers but can also have an impact on near-surface soil areas. In addition to soil samples from prehistoric sites [20–22], samples from historical sites can also provide interesting information about past land use and the ecological changes caused by human activity [23–26]. Examples include the specific compositions of soil bacterial communities from pre- and early industrial mining and smelting sites, from various strata of ancient cities [27,28] or from a pre-industrial tannery and dyeing area in a suburban zone of Jena (Germany) [29].

The analysis of bacterial DNA not only provides information on human influence and environmental conditions in the past but also demonstrates how the imprint of past human activities on soils and microbial communities continues to affect today's ecological situation. For instance, bacteria with high tolerance to heavy metals have been found and cultured from the surroundings of bronze and copper artifacts that had been buried in the soil for centuries. One example is a highly cobalt- and nickel-tolerant strain of *Rhodococcus erythropolis* from a medieval non-ferrous metalworking site [30].

The question of whether the composition of soil bacterial communities can provide information on prehistoric events and objects is especially relevant for so-called “find-poor” structures. Such situation can concern, for example, the defensive Ditches of Neolithic enclosures, which are often low in artifact finds. Understanding their origin and use is crucial for reconstructing life in the corresponding prehistoric periods and for understanding the interplay between climate, land use, and human environmental impact. This applies, for example, to enclosures of the Michelsberg Culture, which are probably linked to intensive livestock farming and long-distance grazing [31,32]. Such practices likely had a significant impact on vegetation at the time and were also fundamentally important for the early development of a road network and the human-shaped structuring of the cultural landscape in Central Europe.

According to radiocarbon dates from finds at various sites, the Michelsberg culture is dated to the relatively long period between approximately 4400 BC and 3500 BC. This means that this culture must have been a determining factor in the cultural landscape development over a substantial part of the Neolithic. It chronologically follows the Rössen culture, which in turn succeeded the Linear Pottery culture, with which the intensive agricultural colonisation of Central Europe began.

The distribution area of the Michelsberg culture extends across central Europe north of the Alps, from the Paris Basin through the German upland regions to the western Bohemian Basin. Thus, the Michelsberg culture is of great importance for prehistoric development in Central Europe not only in temporal but also in spatial terms. The evidence for a larger number of earthworks attributed to the Michelsberg culture in the transitional zone between the uplands and the North German Plain is particularly significant for research into economic practices related to livestock husbandry and probably long-distance transhumance. This activity presumably not only influenced the local formation of paths, but may also have been decisive for the early establishment of long-distance route corridors.

For assessing the function and period of use of Neolithic earthworks that are poor in finds, or as a complement to archaeological evidence, the analysis of DNA from soil bacterial communities can provide additional insights. On the one hand, differences may result from variations in the present-day soil bacterial communities caused by local environmental conditions in the surrounding subsoil and in the fill material of ditches. On the other hand, DNA from dormant bacteria can also provide information on the conditions during the infilling of ditches or even during the phase when the ditch was open. In this respect, deeper ditch sections are of particular interest.

Because of the relatively large spatial extent of earthworks, which typically measure several hundred meters, these features generally cannot be archaeologically investigated across their entire area due to the high effort involved. Instead, individual ditch sections are often excavated in order to characterize the profiles archaeologically. The opening of such sections then makes it possible to take

soil samples from different depths and to examine whether, and which, differences exist in the bacterial DNA of the soil material.

The following presents the results of a study on bacterial DNA extracted from such a Neolithic enclosure Ditch. As part of the profiling of an earthwork at Hachum (Lower Saxony, Germany), 13 soil samples were taken from various positions within the profile. DNA was extracted from these samples, and the composition of the soil bacterial community was determined via NGS analysis of the 16S rRNA gene.

2. Experimental

2.1. Archaeological Situation

The site of the earthwork (UTM coordinates: approx. 614850/5782740) is located about half a kilometer southwest of the village of Hachum in Lower Saxony, Germany, at an elevation of approximately 132 meters above sea level. The earthwork occupies a shallow terrain shoulder, with the land gently sloping to the south and west (situation of Ditch cut in archaeological investigation shown in Figure 1a). The feature was discovered through aerial photography and subsequently investigated archaeologically. Current findings suggest it is a Ditch system from the Neolithic Michelsberg Culture.

During the archaeological investigation, a section of the Ditch was opened and the profile examined. Soil samples were collected from 13 positions. These positions were selected so that some samples were taken from inside the central Ditch, and others from outside or at the edge of this Ditch (Figure 1b). Five samples (Nos. 8, 2, 7, 6, and 3) were collected as a depth profile from the deepest part of the Ditch. Another group of three samples (positions 4, 11, and 12) comes from a structure interpreted as a step or secondary Ditch south of the main Ditch, and thus the soil material from there can also be considered Ditch fill, like the first group. The remaining five samples were taken from outside the inner Ditch structure, with four samples located in a transitional area (nos. 1, 5, 10, and 13), and sample no. 9 taken from the surrounding undisturbed soil near this transition zone. Sample 1 came from the upper part, samples 9 and 5 from the middle part, and samples 10 and 13 from the lower part of the transition area. These five samples were intended as reference samples for comparison with those from the interior of the Ditch profile.

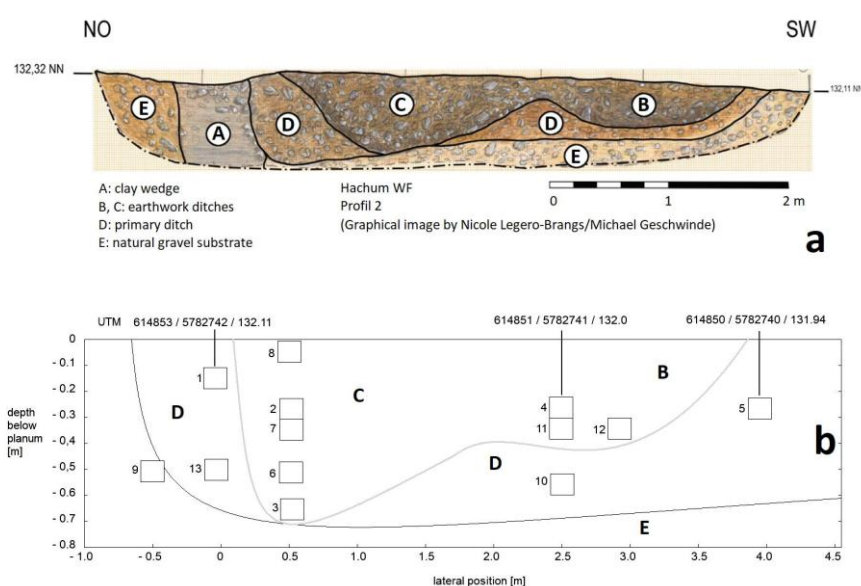


Figure 1. Profile of Neolithic Ditch of Hachum (Germany): a) Situation during archaeological investigation, south part of Ditch cut; b) Scheme of Ditch profile with UTM coordinates and sampling positions.

2.2. Soil Samples and Sequencing

The soil samples were collected using sterile 50 mL sampling tubes and sealed on site. Approximately 1 g of each sample was used for DNA extraction, and a segment encoding the 16S rRNA was amplified by PCR and subsequently sequenced using an Illumina NGS process.

DNA extraction, amplification, and sequencing were carried out by Microsynth using their standard protocol: The samples were first mixed with DNA/RNA shield solution (Zymo Research, Art-Nr.: R1100-250). Then beads 0.1 mm and 0.5 mm were added and a mechanical lysis was realized on TissuelLyzer II. After the mechanical treatment, proteinase K((2 mg/mL; Genaxxon, Art-Nr.: M3036.0500) and RNase A (0.5 mg/mL; PanReac Applichem, Art-Nr.: A3832,0500) were added. The sample are incubated, firstly, at room temperature for ten minutes and for two hours at 56°C. Then DNA was isolated applying Qiagen DNeasy Blood & Tissue Kit (Qiagen, Art-Nr.: 69581). Lumiprobe (fluorescent dye PreciseGreen®, Art-Nr.: 42010) was used for quantification of DNA.

Nextera barcode PCR libraries were generated in a two step process using the locus specific primer pair (515F (5'- GTGYCAGCMGCCGCGTAA-3') and 806R (5'- GGACTACNVGGGTWTCTAAT-3')) for the first PCR followed by a second PCR step for adding the Illumina indices. The sequencing was performed on a NovaSeq 6000 using a 500cy kit in XP mode.

Sample list:

No.	Lab-intern label	depth (below planum)	Lateral position
1	HC1	15 cm	transition region N
2	HC2	25 cm	central Ditch profile
3	HC3	70 cm	central Ditch profile
4	HC4	30 cm	side Ditch
5	HC5	30 cm	transition region S
6	HC6	50 cm	central Ditch profile
7	HC7	35 cm	central Ditch profile
8	HC8	10 cm	central Ditch profile
9	HC9	55 cm	outside Ditch
10	HC10	60 cm	transition region S
11	HC11	40 cm	side Ditch
12	HC12	40 cm	side Ditch
13	HC13	50 cm	transition region N

2.3. Data Processing

The NGS analyses supplied so-called fastq files of sequence data. These data were converted into the format fasta. In addition to this conversion, quality data have been generated by using the open source platform Galaxy (<https://usegalaxy.org/>). The quality of all investigated datasets was checked by a median quality score and found to be high, indicating a very high quality of data.

The taxonomical assignment was achieved by aligning the contig files to rRNA databases based on the NCBI cloud using the SILVAngs data analysis service (<https://ngs.arb-silva.de/silvangs>). This procedure allowed a detailed analysis on the basis of the previously obtained sequencing data, supplying information about the bacterial community of the related sample [33–35]. For all analyses, the preset parameter configurations of the SILVAngs database version 138.2 were applied. In principle, the finally obtained NGS data allow the assignment of 16S rRNA-related DNA down to the genus level. But, a part of cases the assignment is only possible for higher taxonomical levels as families, orders, classes or phyla. Therefore, the determined best assigned taxonomical groups for a sequence were defined as “Operational Taxonomical Unit” (OTU).

3. Results and Discussion

3.1. Composition of Soil Bacterial Communities by Phyla

All samples show a dominance of the phyla *Pseudomonadota* and *Actinomycetota*. *Chloroflexota* and *Acidobacteriota* are also highly represented, although their relative abundances vary significantly between the samples (Figure 2). Samples 2, 6, 7, 11, 12, and 4—all taken from medium depth inside the Ditch—show the highest proportions of *Acidobacteriota* (Figure 3a). The samples from the deepest layers, 3 and 10, have the highest proportions of *Pseudomonadota*, but at the same time show only a negligible proportion of Archaea, whereas the samples from the middle Ditch layers (samples 2, 4, 11, and 12) exhibit the highest *Archaea* proportions. The central part of the Ditch interior (including samples 6 and 7) also displays the relatively highest proportions of *Myxococcota*. The highest abundances were found in the sample pairs 2 and 7 (central Ditch profile), and 4 and 11 (secondary Ditch) (Figure 3b). The next highest proportions occur in samples 6 and 12, which also come from mid-depth layers inside the Ditch. In contrast, both the reference samples and the uppermost sample from the central Ditch profile (sample 8) show considerably lower proportions of *Myxococcota*.

Both *Acidobacteriota* and *Myxococcota* are typical soil bacteria. *Myxococcota* are known for their chemoorganotrophic metabolism, and their high abundance may indicate a high content of organic matter in the soil. The similarity of the samples from the mid-depth layer of the Ditch interior is also reflected in their relatively high content of *Planctomycetota* (Figure 4a) and *Methylomirabilota* (Figure 4b). *Planctomyces* are chemoorganotrophic, facultatively aerobic bacteria typically found in aquatic environments, soil, and sewage sludge. *Methylomirabilota* is a relatively newly identified phylum, known for methane degradation and the coupling of the release of reducing equivalents during methane oxidation with the reduction of nitrite to molecular nitrogen [36].

Differences in the relative abundances of selected groups such as *Archaea*, *Acidobacteriota*, and *Myxococcota* can be interpreted as distinguishing features of the Ditch interior—i.e., the Ditch infill, which must have occurred in prehistoric times—as opposed to the surrounding areas. However, this pattern for the mid-depth zone is somewhat relativized by a comparison of phylum composition between sample 3 (deepest layer of the central Ditch profile) and sample 10 (reference sample from the deeper transitional area), which show a strikingly similar phylum composition.

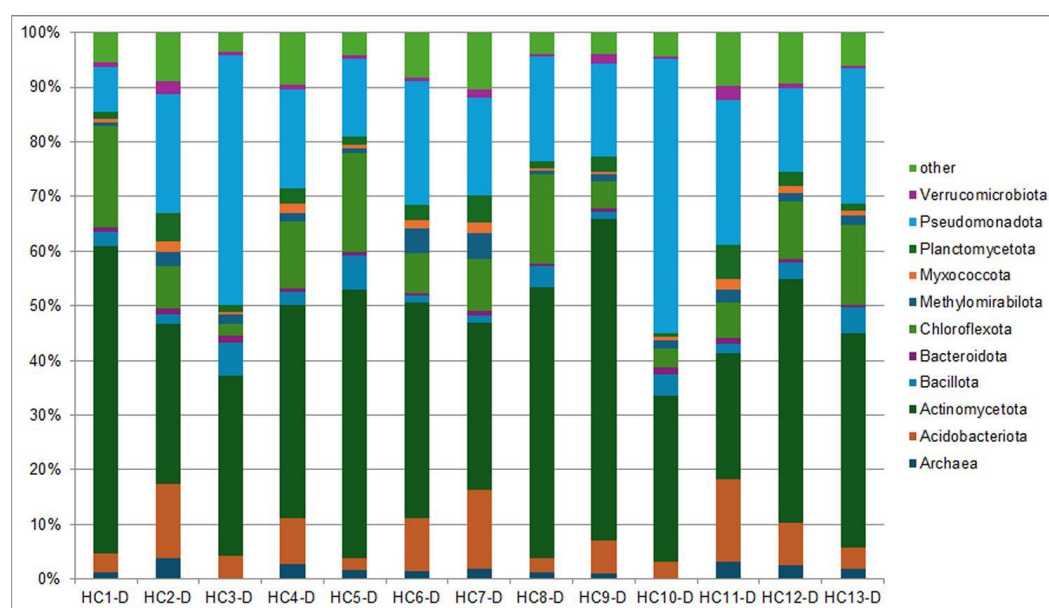


Figure 2. Comparison of phyla abundance (percentages) in the taken samples represented by 16S rRNA.

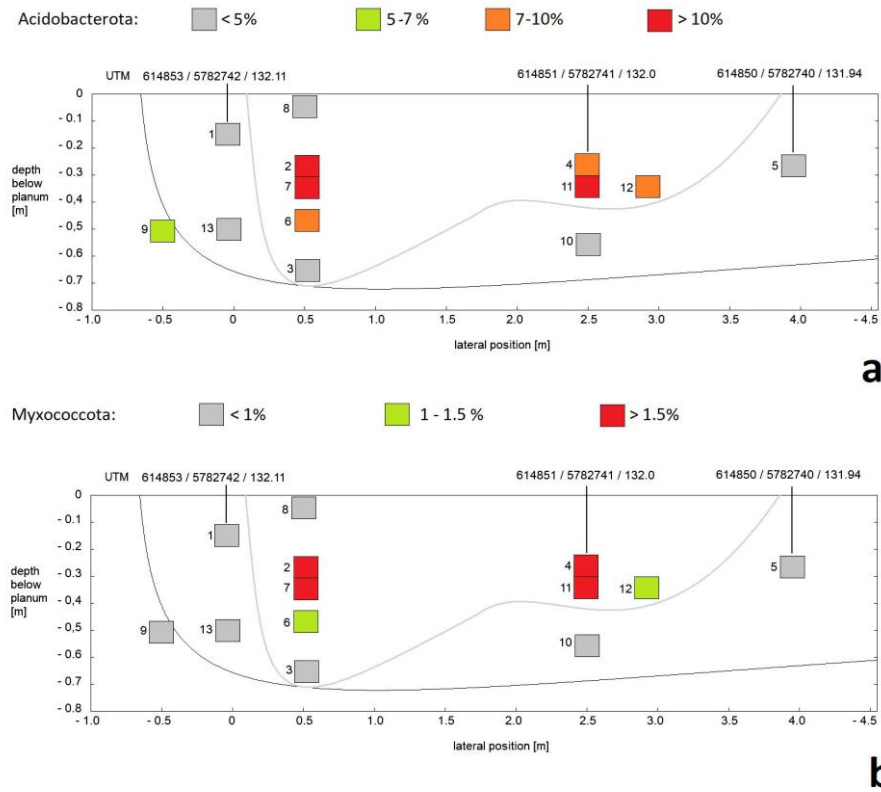


Figure 3. Phyla preferentially present in middle depth inside the Ditch profile: a) Percentage of Acidobacteriota in the Ditch profile b) Percentage of Myxococcota in the Ditch profile.

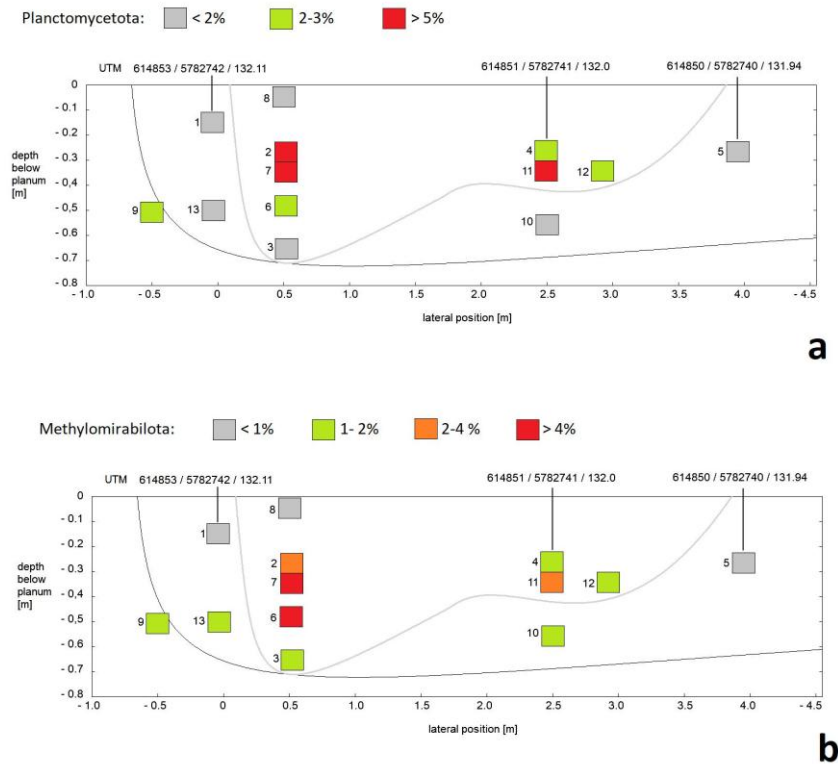


Figure 4. Phyla preferentially present in middle depth inside the Ditch profile: a) Percentage of Planctomycetota in the Ditch profile; b) Percentage of Methylomirabilota in the Ditch profile.

3.2. Composition of Soil Bacterial Communities by Operational Taxonomical Units (OTUs)

While only about a dozen phyla are relevant for comparison between the samples at the high taxonomic level, the sequence data from Hachum's soil samples show a total of around 2000 different OTUs, with hundreds present in each individual sample. Besides individual detections ("reads") that are naturally subject to high uncertainty, there are other OTUs represented by hundreds or thousands of reads in individual samples. Quantitative comparisons of these are suitable for characterizing similarities and differences between the soil samples.

Binary correlation diagrams of read counts for individual OTUs show a high similarity within the group of samples 2, 4, 6, 7, 11, and 12 (examples in Figure 5). These high correlations support the strong similarity in general composition previously derived from the frequent occurrence of certain phyla (see above) in samples from mid-depth layers within the Ditch profile. In contrast, correlations between mid-depth and deep layers, as well as between the Ditch interior and the transitional area, are much weaker (examples in Figure 6). Thus samples 6, 10 and 13 are only weakly correlated despite the fact that they have been taken from a similar depth. The differences can be explained by the different lateral position in the profile. Whereas samples 13 and 16 were originating from outside of the Ditch, sample 6 comes from the central part of the Ditch.

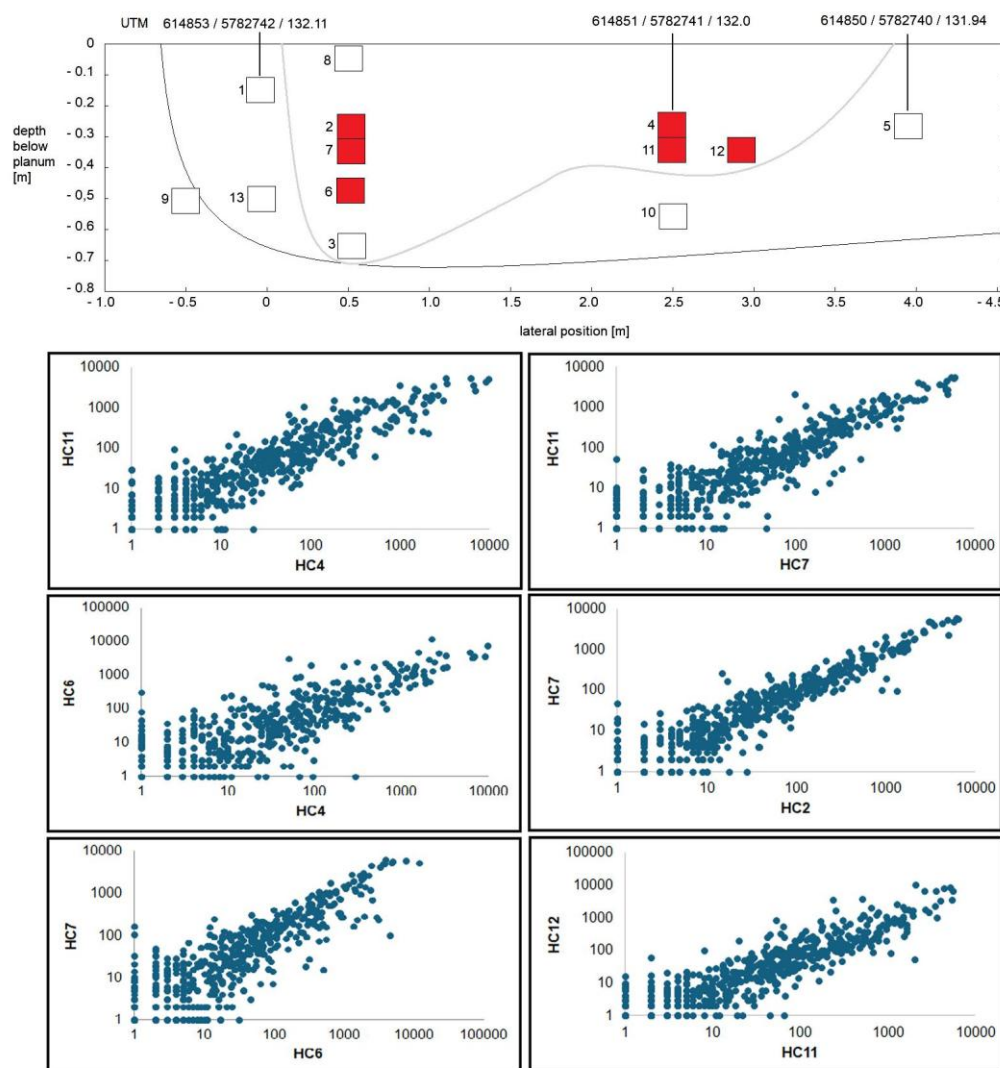


Figure 5. Examples of binary correlation diagrams for the number of reads of all detected OTUs showing a comparatively high correlation between samples taken in middle depth inside the Ditch profile.

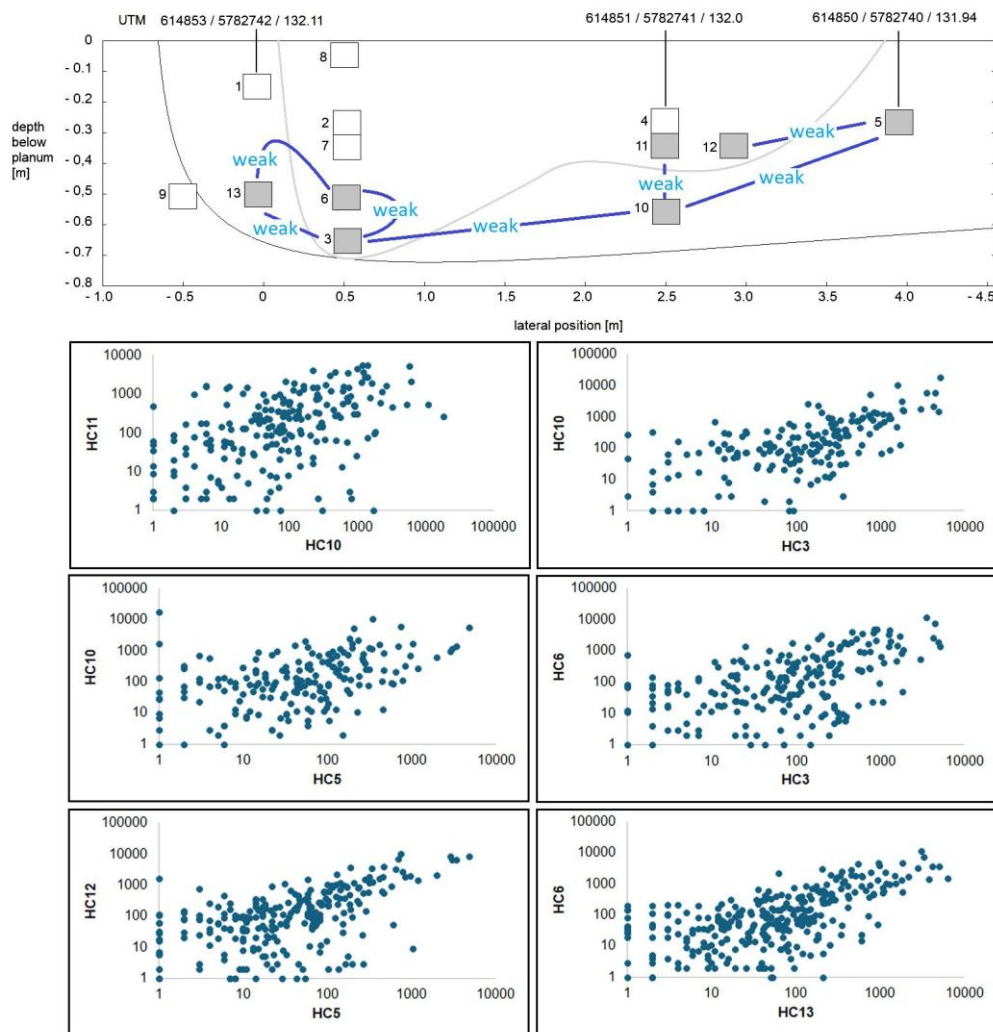


Figure 6. Examples of binary correlation diagrams for the number of reads of all detected OTUs showing a comparatively low correlation between selected samples taken in the central part and in the periphery of the Ditch profile.

The correlation between samples 6 and 3 (central Ditch depth profile) is also very weak, showing that the bacterial community at the bottom of the Ditch differs markedly from that of the overlying Ditch fill. The same applies to the comparison between samples 11 (Ditch interior) and 10 (reference), taken from the same lateral position but slightly different depths. Even the reference samples themselves (outside the Ditch) show only weak correlations. Sample 8 (from the upper part of the Ditch) also shows weaker correlation with the mid-depth Ditch samples.

The similarity among the mid-depth Ditch infill samples (2, 4, 6, 7, 11, and 12), and their distinction from other samples, is also evident when looking at the abundance of individual OTUs—even including less abundant types. There is a larger group of OTUs preferentially found in these samples, while they are much less represented in the upper (sample 8), bottom (sample 3), and reference samples (samples 1, 5, 9, 10, and 13) (Figure 7). This group of OTUs includes *Amaricoccus*, commonly found in sewage sludge [37], as well as *Sandaracinus* [38] and *Sumerlaea*, known for degrading starch and other large carbohydrate molecules [39]. In contrast to other samples from this area, the observed occurrence of bacterial taxa associated with the degradation of starch and other carbohydrates can be interpreted as evidence for the deposition of soil material that, unlike other soil areas, contained increased proportions of plant remains. This may point to a phase of infilling during which the area of the earthwork no longer served as a place for livestock or during which such use at least receded markedly compared to the preceding period. An infilling with comparatively

homogeneously composed material is also reflected in the relatively high similarity of all soil bacterial DNA found in the central part of the ditch interior, as shown both in the correlation diagrams (Figure 5) and in the composition of the specific OTUs (Figure 7).

Differences between sample groups are also reflected in a group of OTUs that occur only in small numbers in samples of the middle part of Ditch 2, 4, 7, 11, and 12, but are comparatively higher abundant in samples outside the Ditch interior, as well as in the top layer (sample 8) and the bottom of the central Ditch profile (sample 3) (Figure 8). This group includes *Brevibacterium*, *Enterobacter*, *Enterococcus*, *Acinetobacter*, *Methylobacterium*, *Methylobacterium*, *Gulbenkiana*, *Duganella*, *Weizmannia*, and *Zoogloea*. *Brevibacterium* and *Weizmannia* have been found, *Brevibacteria* are used in production of cheese [40]. *Enterobacter* and *Enterococcus* are typical gut bacteria. *Acinetobacteria* and *Gulbenkiana* have been found in wastewater [41]. *Methylobacterium* and *Methylobacterium* are soil bacteria that metabolize C1 compounds. These bacteria seem to indicate that the transition region around the Ditch was also affected by dung or waste water infiltration. According to the archaeological situation, the transitional zone could belong to an early phase of partial infilling and restoration of the ditch. The observed composition of bacterial DNA from soil samples taken in these areas can be interpreted as the infilling of soil material that was mixed with residues of animal faeces

The relation to dung and feces is obviously stronger pronounced by OTUs mainly appear in sample 3. The related group of OTUs occurs preferentially in the area of the central Ditch bottom (sample 3), while reads of these OTUs were only sporadically detected in other samples (Figure 9). This underscores the unique nature of the Ditch bottom in comparison to the overlying layers of the central Ditch profile, the rest of the Ditch interior, and the reference samples. These special OTUs include *Fingoldia*, *Eremococcus*, *Coprothermobacter*, *Oryzomicrobium*, *Desulfotomaculum*, *Verrucomicrobium*, *Asaccharospora* and *Hathewayia*. *Eremococcus* has been detected in the genital tract of horses [42], *Coprothermobacter* was isolated from manure [43]. *Oryzomicrobium* is found in muddy soil [44]; *Hathewayia* is a human and animal pathogen. *Desulfotomaculum* can reduce sulfate to hydrogen sulfide using hydrogen and has been found, for example, in deep subsurface layers during deep drilling [45]. *Verrucomicrobia* are comparatively abundant in environment as soils, but were also found in human feces [46]. *Asaccharospora* was isolated from the intestinal tract of rats [47], *Granulicatella* is known as a pathogen, but can also be present in the normal flora of human intestinal tract [48]. The common appearance of these bacteria which are less present in the other samples speak, obviously, for a formerly situation of the open Ditch, in which wastewater, dung and perhaps human feces were accumulated at the bottom of the Ditch.

The sample taken from the ditch bottom records the phase immediately following an evident restoration of the ditch and the use of the earthwork in the directly subsequent period, during which the ditch remained completely open. The indication of a moist environment into which animal and possibly also human faeces, as well as possibly animal remains with elevated sulphur content (fur, horn?), were introduced suggests that the earthwork served as a place for livestock herds during the phase when the ditch bottom was open. The earthwork may have served to protect livestock herds or simply as an enclosed gathering place. Since the construction of such an extensive earthwork required considerable collective effort, its use must have been of great importance for the respective group. If the findings from the ditch bottom are correctly interpreted as indicators of animal faeces, this would also imply that livestock husbandry was of great importance to the builders of the Hachum earthwork.

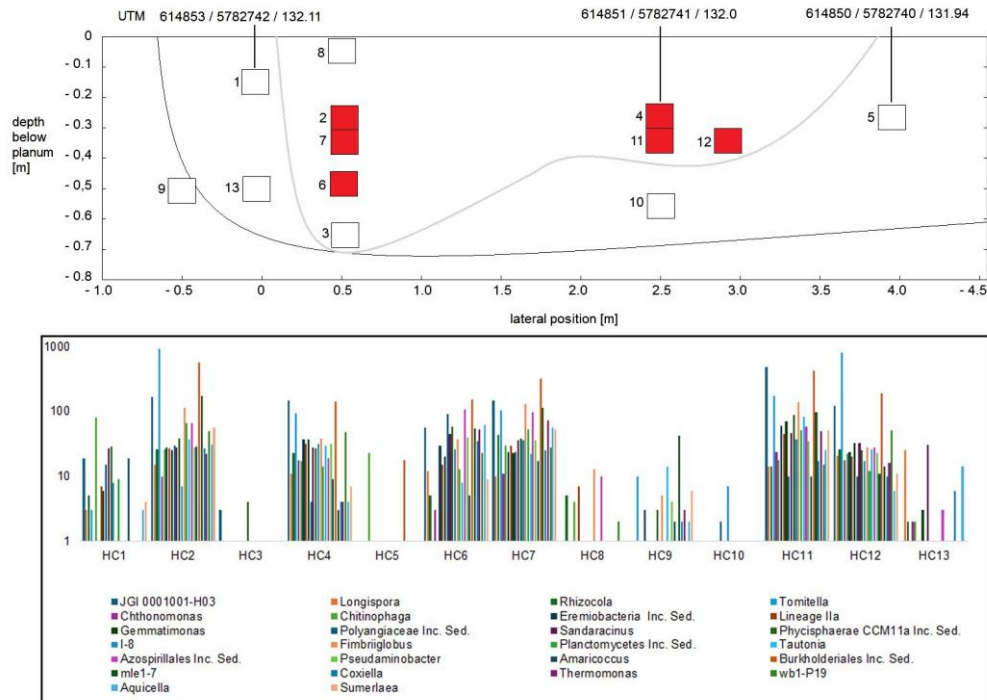


Figure 7. Group of OTUs (number of reads) exclusively or preferentially present in middle depth inside the Ditch profile.

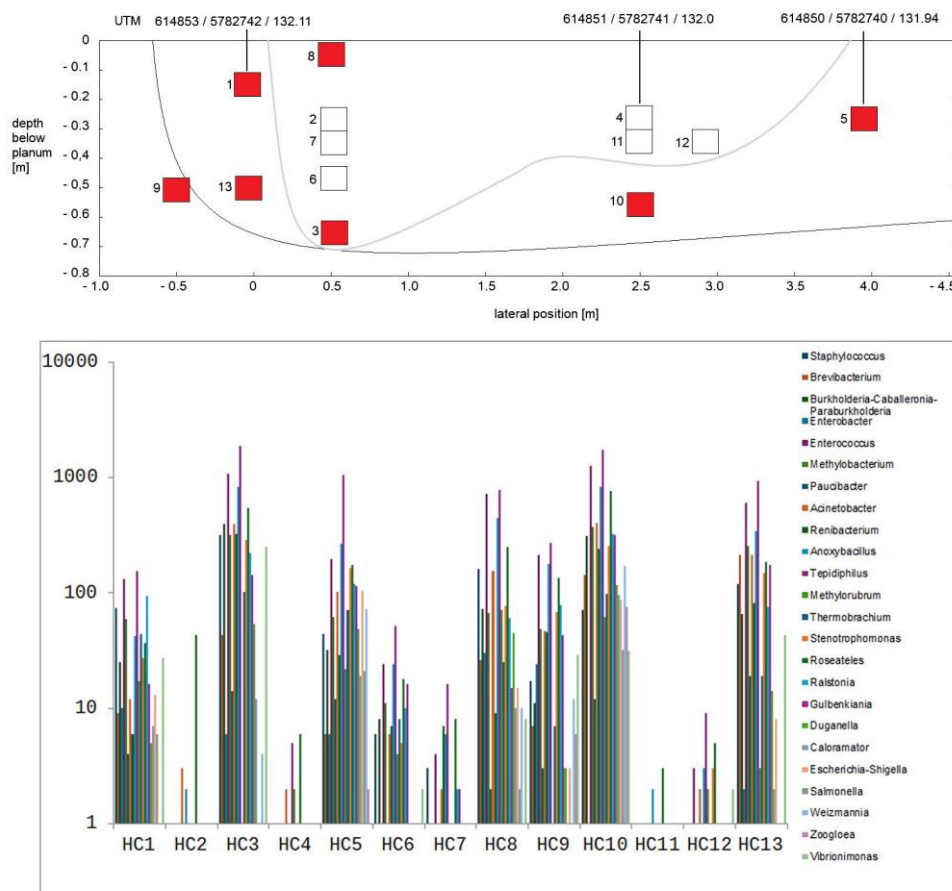


Figure 8. Group of OTUs (number of reads) exclusively or preferentially present outside the sampling positions of the central part inside the Ditch profile.

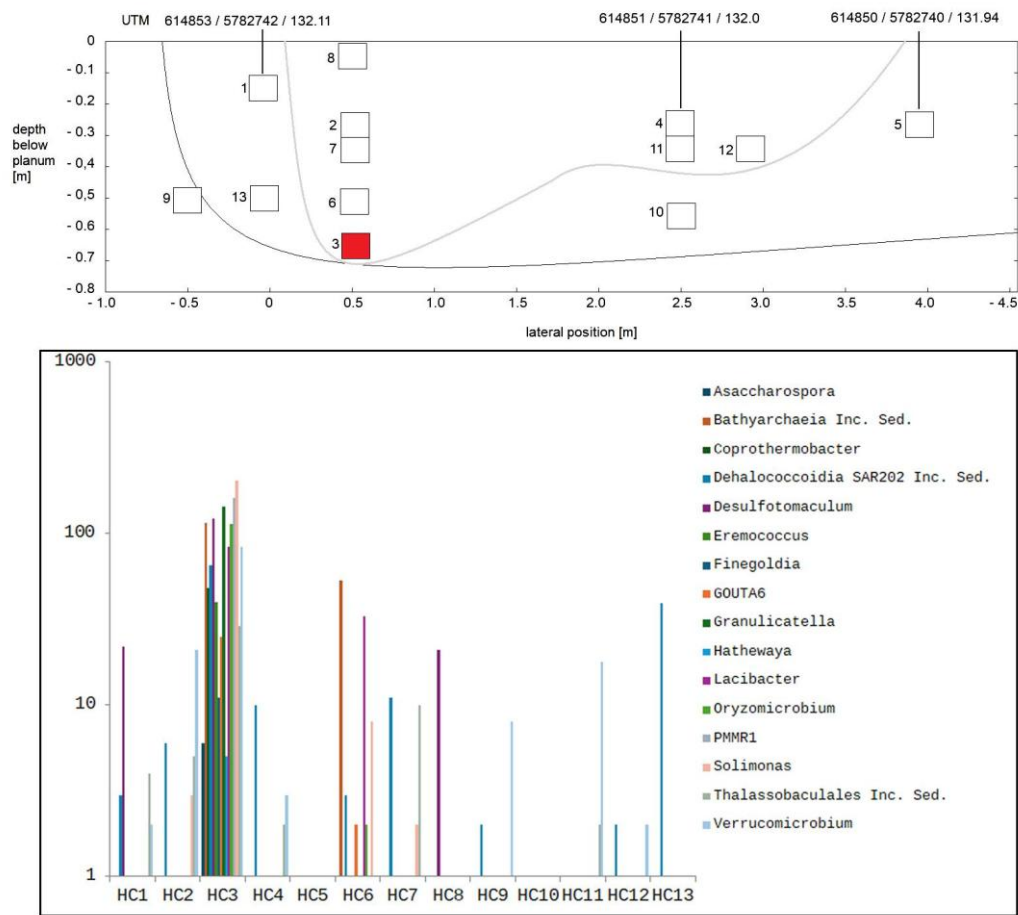


Figure 9. Group of OTUs (number of reads) exclusively or preferentially present at the bottom of Ditch profile.

4. Conclusions

The analysis of the samples taken at different positions of the archaeological profile allows a clear distinction of the observed DNA of soil bacterial communities. In particular, the samples originating from the middle layers of the Ditch are marked by similarities between them and differences to the other samples. These similarities and differences could be shown by comparison of the high taxonomical level of phyla, by binary correlation diagrams of all found OTUs as well as by the presence or, respectively, absence of special OTUs. A particular feature of the filling of this middle region of Ditch could be a significant input of plant materials reflected by bacterial types specialized for metabolization of high molecular weight hydrocarbons.

In addition, a particular specificity of soil bacterial composition was observed in the sample taken from the bottom of Ditch, in which several types are preferentially present which are related to manure and – perhaps human feces. The found group of special bacteria could indicate an accumulation of such sewage from the environment of the Ditch after its construction and before its filling. To the best of our knowledge, the application of NGS of bacterial communities was used the first time for distinguishing soil material from a neolithic earthwork.

In conclusion, the investigations show that the analysis of soil bacterial communities from a profile of a neolithic rampart Ditch could supply information of the ancient situation complementing the archaeological findings and might be particularly valuable for archaeological objects supplying only few or uncharacteristic artefacts.

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