

Article

Microbiological Confinement of Two Adjacent Water Wells in Lake Karla Basin, Greece

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Abstract: We analyzed the bacterial and archaeal community structure of two adjacent irrigation well waters of the Lake Karla Basin, Central Greece, in order to elucidate their connectivity or confinement by using 454 tag pyrosequencing of the 16S rRNA genes. Although considerable overlap was found at the phylum/high taxonomic level, and also at the operational taxonomic units (OTU) level, the dominant, and most likely active, prokaryotes represented by these OTUs were very different between the two wells. As expected, we found higher bacterial species richness compared to that of archaeal, and this renders Bacteria better for the study of connectivity or confinement of water wells. Some of the taxonomic groups found are amongst those found typically in the terrestrial subsurface and also those that have been recently described, enhancing the importance of the subsurface for expanding our knowledge on microbial diversity. The majority of the archaeal and several of the bacterial OTUs, including the most dominant ones in each well, were related to marine or saline environments, indicating the previously suggested persistence of fertilizer residuals in the basin's soils.

Keywords: bacteria; archaea; terrestrial subsurface; water

1. Introduction

Agricultural areas need water for irrigation and one source is lakes and reservoirs, but also groundwater wells. This is one of the reasons that in Eastern Central Greece, a part (*ca.* 38 km² surface area) of the former (*ca.* 180 km² surface area) Lake Karla has been under reconstruction for the past few years after it was anthropogenically dried up in 1962. However, the Lake Karla Basin today, is a typical example of groundwater over-exploitation. It is intensively cultivated with high water demanding crops [1]. Part of the area's irrigation needs is served through an extensive network of pumping wells that cause problems with the basin's water table [2]. It has been estimated that the groundwater level is currently more than 200 m below the land surface at the southern extent of the basin, and the head losses within zones with the most intensive pumping have reached up to 80 m during the last 20 years [3]. This extended network of pumping wells from the same aquifer provides an excellent field for testing connectivity *vs.* confinement in the terrestrial subsurface waters.

Groundwater habitats are known to host immense numbers of prokaryotes compared to surface waters, yet with much lower metabolic activity mostly due to shortage of energy substrates. Although these systems do not show significant temporal variation, their spatial diversity can be important, reflecting differences in available substrates [4]. To date, there are no taxonomic groups that could be considered as typical for undisturbed groundwater systems [4,5]. On the other hand, microbial community characteristics provide more insights into ecosystem confinement, functionality, and even health. For example, in the marine environment, some biodiversity features of marine bacteria, such as species distribution and community structure, have been proposed as indicators of marine health status [6]. Likewise, Meziti *et al.* [7] used bacterioplankton community temporal and spatial variability in a semi-enclosed system in order to elucidate impacts from freshwater inputs from the land or a near-by sewage treatment plant. Finally, Pachiadaki and Kormas [8] used co-occurrence of bacterial and archaeal phylotypes in distant methane seeps for depicting isolation *vs.* inter-connectivity of these deep-sea benthic habitats.

Based on cross-ecosystem investigations, it has been shown that the higher phylogenetic dissimilarities occur between different habitat types than within them, and this is more obvious for subsurface habitats [9]. For instance, deep water and subsurface ecosystems are considered to be relatively stable with the spatial and temporal variation of their microbial communities governed not only by geophysical features but also by hydrochemical and biotic factors [10,11]. Based on these considerations, in this paper, we hypothesized that if two adjacent water wells are connected, then more than 10% [12] of their prokaryotic community structure would be similar. We used bacterial and archaeal community structure to provide evidence for the confinement or connectivity of two adjacent water wells used for irrigation purposes in the Lake Karla Basin, Greece.

2. Materials and Methods

2.1. Sampling

Water from two adjacent water wells, named KSL (39°27'13.96" N, 22°45'07.15" E) and KSP (39°26'39.92" N, 22°44'54.93" E) from the Lake Karla Basin, Central Greece, was collected on 15 September 2011. The depth of the wells, which are 1096 m apart, was 68 and 162 m below surface

(mbs) for KSL and KSP, respectively (Figure S1). The basic abiotic parameters of the two wells along with the prokaryotic abundance of their waters indicates they are very similar environmental settings (Table S1). From each well, the water was collected in sterile 10 L carboys after letting the water flow at its maximum speed for 10 min. Each carboy was kept in darkness and chilled in a foam box and was transferred to the laboratory within 3 h. Approximately 9 L from each well were pre-screened through a 180 μm nylon filter (Millipore, Billerica, MA, USA). The filtrate was filtered through a 0.2 μm isopore polycarbonate filter (Sartorius, Göttingen, Germany) and stored immediately at $-80\text{ }^{\circ}\text{C}$ until further analysis.

2.2. Molecular Analysis

The stored filters were aseptically sliced and their bulk DNA was extracted using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA concentrations ranged from 6.2 to 16.1 $\text{ng}\cdot\mu\text{L}^{-1}$. The 16S rRNA genes were amplified with bacterial primers 27f (AGRGTTCGATCMTGGCTCAG) and 519r (5'-GTNTTACNGCGGCKGCTG-3') and with archaeal primers 349f (5'-GYGCASCAGKCGMGAAW-3') and 915r (5'-GTGCTCCCCCGCCAATTCCT-3'). Sequencing was performed at the sequencing facilities of MRDNA Ltd. (Shallowater, TX, USA). Amplicon pyrosequencing (bTEFAP) was performed as described in Dowd *et al.* [13]. In brief, a one-step 30-cycle polymerase chain reaction (PCR) reaction was applied using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). PCR conditions included: 94 $^{\circ}\text{C}$ for 3 min, followed by 28 cycles of 94 $^{\circ}\text{C}$ for 30 s; 53 $^{\circ}\text{C}$ for 40 s and 72 $^{\circ}\text{C}$ for 1 min; and a final elongation step at 72 $^{\circ}\text{C}$ for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX Titanium instruments and reagents following the manufacturer's guidelines.

2.3. Data Processing and Analysis

Sequence data were analyzed using the software MOTHUR 1.33.0 [14]. Flowgrams from each sample were separated based on their tag and then denoised using PyroNoise software [15]. After removing primer sequences, TAG, and key fragments, only sequences with ≥ 200 bp with homopolymers < 8 bp were considered for further analysis. Chimeric sequences were removed using UCHIME [16]. Clustering of the remaining sequences was based on a 97% similarity cut-off limit. Singletons, *i.e.*, sequences that occurred only once in the whole dataset, were removed from further analysis, as they are likely to be sequencing artifacts [17,18]. Taxonomic affiliation was assigned according to the SILVA 111 SSU RNA database [19]. Sequences from this study have been submitted to the GenBank Short Reads Archive (accession number SRX1181212).

3. Results and Discussion

We used 16S rRNA gene tag pyrosequencing to reveal the bacterial and archaeal community structure of two adjacent water wells as a proxy for determining the wells' degree of connectivity or confinement. We hypothesized that if the wells were connected, the circulating water in those wells

would harbor microbial communities of similar phylogenetic diversity and structure. Rarefaction analysis showed our sequencing efforts reached a plateau (Figure 1), indicating that our analysis revealed the majority of the species richness present, *i.e.*, operational taxonomic units (OTUs), for both the Bacteria and the Archaea. This was also depicted in terms of sample coverage (Table 1), which exceeded 91.8% for both samples and both domains of life. This shows the 454 pyrosequencing method was able to satisfactorily reveal the species richness of these aquifers [20] compared to earlier 16S rRNA gene clone libraries and sequencing [21].

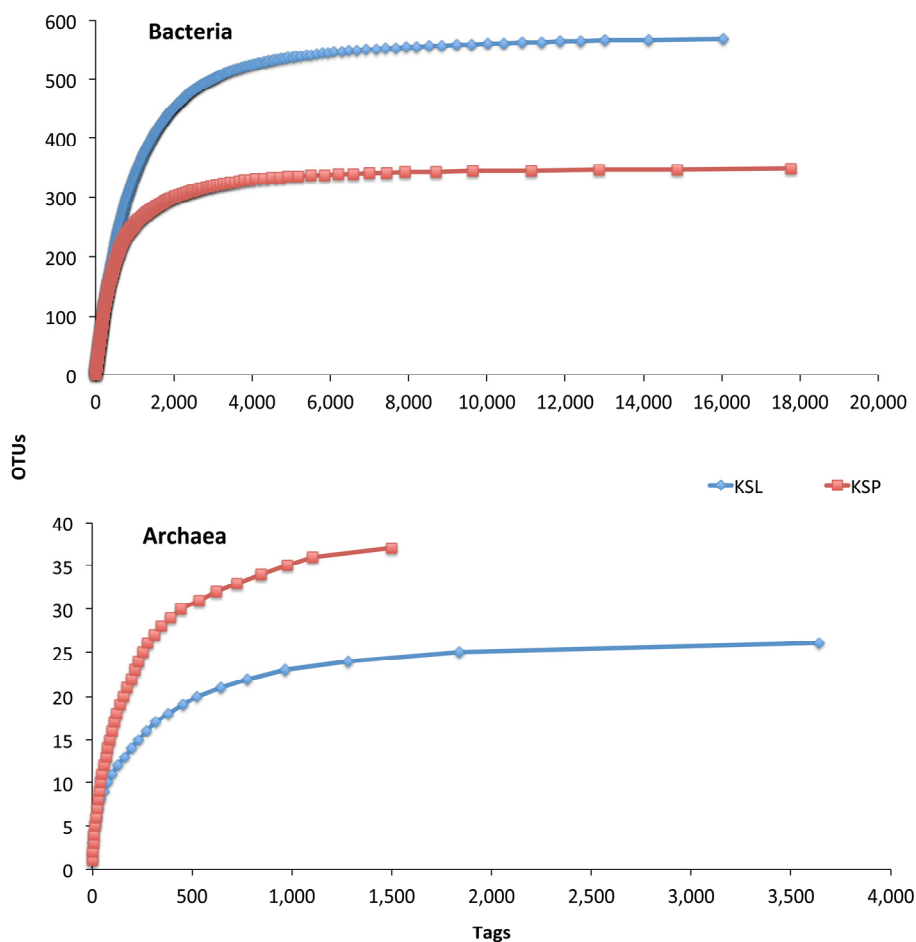


Figure 1. Rarefaction curves of the operational taxonomic units (OTUs) in two water wells (KSL and KSP) of the Lake Karla Basin, Greece.

Table 1. Pyrosequencing results of the 16S rRNA gene in two water wells (KSL and KSP) of the Lake Karla Basin, Greece. OTUs: operational taxonomic units.

Sample	Reads	OTUs ¹	Coverage (S _{Chao})	Dominance of the Most Abundant OTU	No. of Most Dominant OTUs (Cumulative Relative Dominance)
Bacteria					
KSL	16,529	568	91.8%	11.7%	29 (66.0%)
KSP	18,056	348	92.6%	16.0%	11 (66.0%)
Archaea					
KSL	3,637	25	99.4%	49.5%	3 (73.4%)
KSP	1,500	36	93.9%	26.4%	8 (73.9%)

Note: ¹ singletons excluded.

Bacterial OTU numbers were *ca.* 23 and 10 times higher compared to those of the Archaea for KSL and KSP, respectively. It has been shown, for a variety of aquatic environments, that archaeal communities have lower species complexity compared to the Bacteria [22], possibly due to the constant environmental stress the Archaea face [23]. The first evidence for possible connectivity between the two wells arises from their similar fundamental abiotic and biotic features (Table S1). The high percentage of shared (common) OTUs between the two wells may support this notion (Figure 2). The two wells share 15.4% of the 794 unique bacterial OTUs but 34.4% of the 40 unique archaeal OTUs. Although no globally accepted threshold of shared OTUs between two habitats exists that defines connectivity *vs.* isolation, it has been suggested that when habitats have <10% of shared bacterial types, then they are considered rather distinct, *i.e.*, isolated from each other [12]. Shabarova and Pernthaler [24] reported only one common OTU out of a total of 150 OTUs in three subsurface pools of the same karst cave system. Taking into account the proximity of the two wells, the rather high percentage of shared OTUs may indicate close connection between them, and/or similar dominant environmental parameters.

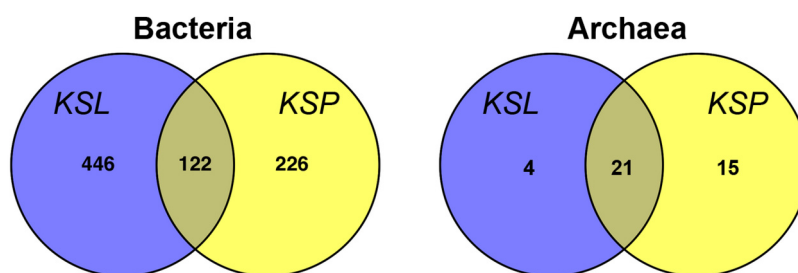


Figure 2. Venn diagrams of the shared and unique bacterial and archaeal operational taxonomic units in two water wells (KSL and KSP) of the Lake Karla Basin, Greece.

The next evidence for possible connectivity between the two wells comes from the community structure at the two sites. Regarding Bacteria, the 794 OTUs belonged to 31 higher taxonomic groups, with 24 of these groups being found in both wells. However, the relative abundance of these taxa was different in the two wells. The five most abundant taxa (Figure 3) for KSL were the *Candidatus Saccharibacteria* (15.5%), *Verrucomicrobia* (12.0%), *Gracilibacteria* (7.8%), *Actinobacteria* (6.5%) and *Chlamydiae* (5.9%), while the five most dominant taxa for KSP were the *Planctomycetes* (32.4%), *Verrucomicrobia* (8.3%), *Latescibacteria* (7.9%), *Acidobacteria* (6.6%) and *Candidatus Saccharibacteria* (6.3%). Members of these groups have been detected previously in subsurface habitats [5,25,26], reflecting the subsurface character of our samples. All archaeal OTUs belonged to the *Euryarchaeota*, *Crenarchaeota* or were unaffiliated. Due perhaps to the small number of archaeal OTUs detected, no obvious differences in the relative abundance of the higher archaeal taxa were observed (Figure 3). Although community structural diversity does not necessarily imply functional diversity [27], the differences in the dominant phyla (Figure 3) suggest that the ecophysiological roles of these dominant Bacteria (Table S2) are different in the two wells.

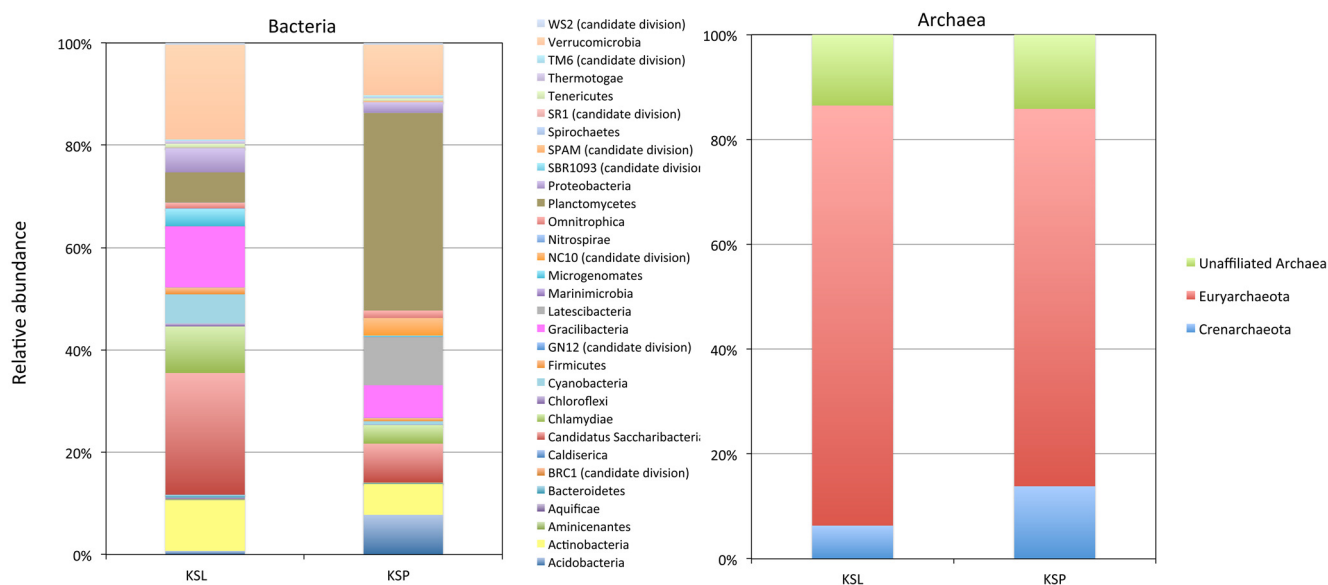


Figure 3. Proportions of the bacterial and archaeal phyla and high taxonomic groups found in two water wells (KSL and KSP) of the Lake Karla Basin, Greece.

Difference between the microbial communities is also evident at the level of the 97% OTUs. Although the two wells share 31 of 35 bacterial OTUs with >1% relative abundance (Figure 4), the relative abundance of these OTUs is also very different between them. For example, the most dominant OTU in KSL was B-001 (11.7%) while in KSP it was B-012 (16.0%). In microbial communities, the dominant species most likely occupies a fundamental niche in that habitat [28]. In KSL, B-001 is related (Table S2) to the marine obligate oligotrophic ultramicrobacterium *Sphingopyxis alaskensis* (fam. Sphingomonadaceae) [29,30] while B-012 is closely affiliated (Table S2) to a Rhodobacteraceae *Thioclava* sp., two bacteria with different cell morphologies, ecophysiological traits and evolutionary histories. The genus *Thioclava* consists of two marine species, *T. dalianensis* [31] and *T. pacifica* [32] both able to oxidize reduced sulfur compounds. A similar pattern was observed for the Archaea (Table S2), despite their much lower species richness. KSL was dominated (49.5%) by a member of the methanogenic Methanosarcinales, while KSP was dominated (26.4%) by a *Nitrosopumilus*-like OTU. The Nitrosopumilaceae family contains three species, all of which have a marine origin [33–35]. The majority of the archaeal OTUs, along with some of the bacterial ones, including the dominant bacterial OTUs B-001 and B-012, are related to taxa from marine or saline environments (Table S2).

The conductivity of the well waters is rather high (Table S1) and seawater intrusion in the basin area has been proposed [36] due to lowering of the water table. However, Sidiropoulos [37] suggests that the increased salinity in the basin's groundwater is mostly due to leaching of salts from the fertilized agricultural land. It has been shown that nitrogen fertilizers applied to crops can stay in the soil and leach out as nitrate to subsurface waters for several decades following their application [38,39]. The increased salinity of Lake Karla water due to such application of fertilizers for several decades prior to its reconstruction might have led to the occurrence of salt tolerant protists and algae during the first years of the Lake's refilling [40].

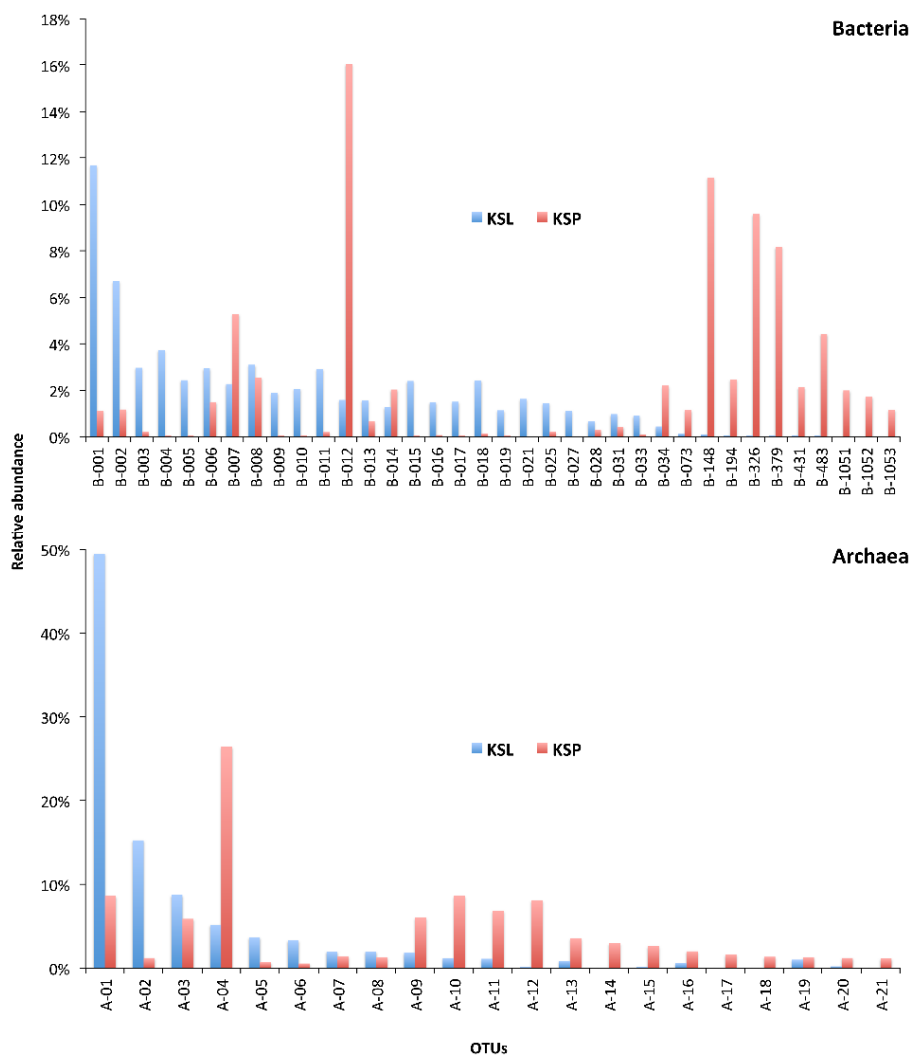


Figure 4. The most dominant bacterial and archaeal (relative abundance >1%) operational taxonomic units (OTUs) in two water wells (KSL and KSP) of the Lake Karla Basin, Greece.

It has been suggested for several years that Earth's terrestrial subsurface biosphere hosts 40%–50% of the world's biomass [41]. Moreover, the microorganisms found in this habitat are frequently novel either at the phylogenetic level and/or their physiological features [42–44]. In this study, we found 31 bacterial phyla or high yet-unaffiliated taxonomic groups. Among these, some have been known previously from subsurface habitats but have only recently been characterized, e.g., Gracilibacteria [45], Candidatus Saccharibacteria [46], Omnitrophica [45], and Microgenomates [45]. Establishment of the taxonomic affiliation of these phyla will enable us to explore their potential ecophysiological role in the subsurface environment. This will require focused and interdisciplinary approaches that fall out of the scope of this paper.

Evidence that the prokaryotic communities in these two wells may be somewhat environmentally/ecologically confined, comes from the rest of the OTUs detected in each well. Subsurface habitats are characterized by high numbers of rare OTUs (*sensu* [47,48]) and this was true for the Bacteria in water samples from these two wells. In KSL, the rare bacterial OTUs comprised 66.9% of the total OTUs while in KSP they comprised 88.2% (Figure 5). Additionally, less than 5% of

the rare OTUs occurred in both wells (data not shown), suggesting that the two wells are located in distinct environmental settings.

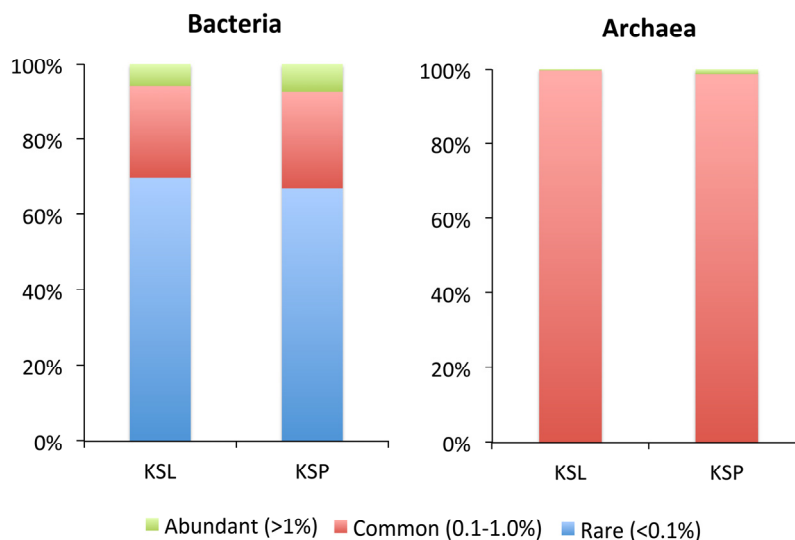


Figure 5. The percentage of abundant, common and rare bacterial and archaeal operational taxonomic units (OTUs) in two water wells (KSL and KSP) of the Lake Karla Basin, Greece.

4. Conclusions

Overall, we showed that adjacent water wells can harbor a high percentage of shared OTUs, however the distribution and structure of their microbial communities shows that these closely-located sites may represent distinct habitats with different environmental conditions. Moreover, the phylogenetic diversity of some OTUs points to long-time persistence of increased salt concentrations at both sites, possibly due to past soil fertilization practices that have led to the occurrence and dominance of some salt-tolerant taxa, mostly Archaea.

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Author Contributions

Konstantinos Ar. Kormas conceived and designed the study; Konstantinos Ar. Kormas, Theodoti Papadimitriou performed sampling and part of the analysis; Konstantinos Ar. Kormas, Alexandra Meziti, Theodoti Papadimitriou performed molecular analysis, data processing and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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