



**PEDRO MIGUEL
PINHEIRO SARAIVA**

**Diversidade bacteriana em águas subterrâneas do
Maciço Calcário Estremenho (Portugal) reveladas por
pirosequenciação**

**Bacterial diversity in groundwater samples from
Estremenho Karst Massif (Portugal) revealed by 16S
rDNA pyrosequencing**



**PEDRO MIGUEL
PINHEIRO SARAIVA**

**Diversidade bacteriana em águas subterrâneas do
Maciço Calcário Estremenho (Portugal) reveladas por
pirosequenciação**

**Bacterial diversity in groundwater samples from
Estremenho Karst Massif (Portugal) revealed by 16S
rDNA pyrosequencing**

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Daniela Rebelo de Figueiredo, Investigadora em Pós-Doutoramento do Departamento de Biologia e CESAM, Universidade de Aveiro, do Doutor Nelson José Cabaços Abrantes, investigador Auxiliar do Departamento de Ambiente e CESAM da Universidade de Aveiro, e do Doutor Daniel Francis Richard Cleary, investigador Principal do Departamento de Biologia e CESAM da Universidade de Aveiro

o júri

presidente

Prof. Doutora Maria de Lourdes Gomes Pereira
Professora associada c/ Agregação, Departamento de Biologia,
Universidade de Aveiro

Doutor Francisco José Riso da Costa Coelho
Investigador em Pós-Doutoramento, Departamento de Biologia e CESAM
da Universidade de Aveiro

Doutora Daniela Rebelo de Figueiredo (orientadora)
Investigadora em Pós-Doutoramento, Departamento de Biologia e
CESAM da Universidade de Aveiro

agradecimentos

Queria agradecer a Daniela Figueiredo, orientadora principal, pela confiança depositada e pelo convite para continuar o trabalho desenvolvido durante a minha Pesquisa de Licenciatura. Mas também pela forte motivação e palavras que me fizeram continuar e incentivaram a ir cada vez mais longe nesta incrível aventura, que é a investigação.

Agradecer também ao Doutor Nelson Abrantes, coordenador do projeto Karatrisk, que permitiu que esta dissertação fosse possível e pela correção da mesma. And Dr. Daniel Cleary for the introduction to QIIME.

Queria agradecer à Dona Celeste, pela simpatia, empenho e avisos antes e durante todo o processo de submissão das provas de dissertação.

A todas as pessoas que tiveram direta ou indiretamente um forte impacto no meu percurso académico e pessoal, nem sempre fácil de enumerar todas elas, fica por isso um agradecimento a todas elas.

Ao Alexandre Ramos, Marta Morais, Pedro Gomes, Soraia Lopes, Inês Gregório e Barbara Cartagena pela amizade e por todos os bons momentos que moldaram a minha personalidade e forma de ser. E que ainda não tenham tido uma ligação direta ao trabalho desenvolvido nesta tese foram essenciais para manter a minha sanidade mental durante todo esta fase de Mestrado. E que por mais distante e diferentes percursos seguidos por cada um, continuamos a partilhar esta amizade.

Por fim agradecer a minha família por todo o suporte e apoio ao longo destes sete anos (que já começam a pesar), suporte imprescindível para o percurso realizado e acima de tudo sem nunca questionarem ou limitarem as minhas aspirações e decisões mesmo sem muitas vezes as entenderem, um MUITO OBRIGADO.

palavras-chave

Comunidades Bacterianas; Maciço Calcário Estremenho; Águas subterrâneas; 16S rDNA-DGGE, Pirosequenciação.

resumo

A água subterrânea constitui uma importante fonte de água doce, que muitas vezes é sobre explorada e impactada pela poluição superficial. É por isso, de grande interesse a compreensão deste ambiente.

Neste sentido, o presente estudo pretendeu analisar pela primeira vez a composição e diversidade de comunidades bacterianas presentes nas águas subterrâneas do Maciço Calcário Estremenho (Centro-Oeste Portugal), através de abordagens moleculares independentes de cultura DGGE e Pirosequenciação.

Os resultados revelaram que este ambiente em particular é geralmente dominado pelo filo Proteobacteria (61,83%) com especial relevância para as ordens Thiobacterales, Rhodocyclales, Burkholderiales e Neisseriales (Betaproteobacteria); Sphingomonadales (Alphaproteobacteria) e Xanthomonadales, Acidiferrobacterales (Gammaproteobacteria). Entre outros filós com menos representatividade como Bacteroidetes (Sphingobacteriia), Actinobacteria (Acidimicrobiia), Acidobacteria, Firmicutes (Bacilli), Elusimicrobia (Elusimicrobia), Gemmatimonadetes, todas elas presentes normalmente em águas doces.

Os resultados de ambas as abordagens moleculares mostraram um agrupamento semelhante observado para algumas amostras, caracterizado por uma influência direta dos ambientes superficiais e indicando um impacto de fontes de poluição, corroborado pelos taxa dominantes nessas amostras: gêneros Limnohabitans e Sphingopyxis e membros da ordem Sphingobacterales, normalmente relacionadas com águas superficiais e poluídas. Estes dados sugerem um impacto direto do uso de terras em comunidades de bactérias de águas subterrâneas. Este trabalho assume-se como o primeiro estudo na determinação da composição e caracterização das comunidades bacterianas de um dos maiores e mais importantes sistemas cársicos da Península Ibérica.

keywords

Bacterial Communities; Estremenho Karst Massif; Groundwater; 16S rDNA-DGGE, pyrosequencing

abstract

Groundwater provides an important freshwater source, that is many times overexploited and suffer pressures from superficial pollution. Therefore, it is of major interest to go further in the understanding of these environments. The present study examined, for the first time, the composition and diversity of bacterial communities present in groundwater from the Estremenho karst massif (Central-Western Portugal), through culture-independent molecular approaches, DGGE and pyrosequencing based on 16 rDNA sequences).

Results showed that this particular environment was generally dominated by Proteobacteria (61.83%), with special relevance to Thiobacteriales Rhodocyclales, Burkholderiales and Neisseriales (Betaproteobacteria) Orders; Sphingomonadales (Alphaproteobacteria) Order, Xanthomonadales and Acidiferrobacterales (Gammaproteobacteria) Orders. Other less abundant phyla included the Bacteroidetes, (Sphingobacteriia), Actinobacteria (Acidimicrobiia), Acidobacteria, Firmicutes (Bacilli), Elusimicrobia (Elusimicrobia), Gemmatimonadetes all normally present in freshwaters.

Results from both molecular approaches showed a similar clustering observed for some samples, characterized by a direct influence from surface environments and indicating an impact from pollution sources, corroborated by the dominant taxa in those samples: genera Limnohabitans and Sphingopyxis and members of the order Sphingobacteriales, commonly related to superficial and polluted waters. These data suggest an interaction of direct impact of surface land use/land cover on groundwater bacterial communities.

This study is the first research for the determination of the composition and characterization of the bacterial communities from one of the biggest and most important karst massif in Iberian Peninsula.

List of communications linked to the present work

Poster communications:

- de Figueiredo D.R., Cleary D., Saraiva P., Gomes N., Gonçalves A.M.M., Reboleira A.S., Gonçalves F., Oliveira J., Condesso de Melo M.T., Abrantes N. (2014) Metagenomic overview of bacterioplankton diversity from Estremenho karst massif aquifer (Portugal). *EcoHCC'14 - 3rd International Conference on Ecohydrology, Soil and Climate Change, Tomar, Portugal*;
- de Figueiredo Daniela R, Saraiva P., Melo Maria T. C, Oliveira J., Gonçalves A.M.M., Reboleira Ana S. P. S, Abrantes N. (2013). Bacterial diversity as a tracker to assess connections and pollution impacts through the aquifer of Estremenho karst massif, *III Encuentro Ibérico de Biología Subterránea, Barcelona, Spain*
- Abrantes N., de Figueiredo D.R., Rosa I.C., Saraiva P., Gonçalves A.M.M., Bessa M., Reboleira, A.S., Gonçalves, F. (2013) Bacteria as a key-community to track variations on karstic aquifers, *SETAC Europe 23rd Annual Meeting, Glasgow, Scotland*;

Index

Index	ii
List of Acronyms	iv
List of Figures	v
List of Tables	vii
1. Introduction	1
1.1. Dissertation Outline.....	1
1.2. Scope, motivation and relevance	1
1.3. Dissertation aims	4
1.4. State of the Art	5
1.4.1. Karst areas and aquifers.....	5
1.4.2. Bacterial communities in groundwater from karst environments ...	10
1.4.3. Metagenomics approaches for the study of microbial communities	11
2. Materials and Methods	15
2.1. Sampling	15
2.2. DNA extraction	15
2.3. PCR-DGGE	18
2.3.1. PCR amplification of bacterial 16S rDNA fragments	18
2.3.2. Denaturing gradient gel electrophoresis (DGGE).....	18
2.4. Pyrosequencing	19
2.4.1. Sequencing	19
2.4.2. Sequences analysis using QIIME	20
2.4.3. Statistical analyses	21
3. Results and Discussion	22
3.1. PCR.....	22
3.2. 16S rDNA-DGGE band patterns and cluster analysis.....	23
3.3. Pyrosequencing	27
4. Conclusion	41

5. References	43
6. Annex	52
6.1. Annex Table 1	52
6.2. Annex Table 2	53
6.3. Annex table 3.....	54

List of Acronyms

DGGE	Denaturing Gradient Gel Electrophoreses
QIIME	Quantitative Insights Into Microbial Ecology
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
OTU	Operational Taxonomic Unit
APS	Adenosine-5'-phosphosulfate
ATP	Adenosine Tri-phosphate
PPi	Pyrophosphate
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
UV	Ultra Violet
WFD	Water Framework Directive
RH	Hydrographical regions (PT)
EU	Europe Union

List of Figures

Figure 1 – Karst landscapes outcrops worldwide with subcrop mapped. Solid colour indicates that carbonates are relatively continuous;.....	6
Figure 2 – Representation of Hydrographic regions in Portugal (adapted from INEG, 2005).....	7
Figure 3 - Major Karst areas (adapted from (Reboleira et al. 2011)).....	8
Figure 4 – Land use/land cover in Estremenho Karst Massif	9
Figure 5 – Collection sites in Serra de Aire e Candeeiros (Maciço Calcario Estremenho) and representation of land cover. At red the urban areas; Yellow the agricultural areas; Green the forest areas.	17
Figure 6 – Agarose gel (1,5%) electrophoresis of PCR products; Samples K1 to K30; Molecular size marker is marked with the letter M the Negative with the letter N	22
Figure 7 - PCR-DGGE profiles containing the bacterial 16S rDNA fragments from water samples k1, k2, k3, k4, k6, k8, k9, k13, k15, k18, k19, k20, k21, k24, k30 collected at Estremenho Kast Massif during February 2012	23
Figure 8 – Bray-Curtis similarity. Cluster dendrogram, with slicing at 40% similarity.	25
Figure 9 – Metric Multidimensional scaling (MDS) with resemblance level of 35%. Formation of 3 groups A, B and C.....	25
Figure 10 –Cluster showing OTU similarity between site samples (relevance >1%) with Bray-Curtis distance.....	28
Figure 11 - Metric Multidimensional scaling (MDS) with resemblance level of 35%. The sample K15 was excluded.....	28
Figure 12 - Rarefaction curves of the 13 samples. The error bars show 95%confidence of upper and lower limits.....	29
Figure 13 – Distribution of the major phyla (with more than 3%) and unclassified OTUs at phylum level. The 6 groups attending, represent 83,81% of all the phyla in the samples collected.	30
Figure 14 - Distribution of the major Classes (with more than 3%) unclassified. The 4 groups attending, represent 61,76% of all the classes in the samples collected	31

Figure 15 - Distribution of the major Orders (with more than 3%) and unclassified OTUs at Order level. The 5 groups attending, represent 24,38% of all the Orders in the samples collected. 32

Figure 16 - Distribution of the major Families (with more than 2%) and unclassified OTUs at Family level. The 5 groups attending, represent 18,14% of all the Families in the samples collected. 33

Figure 17 – Relative abundance of Phylum (with $\geq 1\%$ representation per sample). 34

Figure 18 - Relative abundance of Class (with $\geq 1\%$ representation per sample). .. 35

Figure 19 - Relative abundance of Order (with $\geq 1\%$ representation per sample).. 35

Figure 20 - Relative abundance of Family (with $\geq 1\%$ representation per sample). 35

List of Tables

Table 1 - Consumption values of surface and groundwater in Portugal. Adapted from (Ribeiro & Cunha, 2010)	2
Table 2 - Distribution of bacteria in caves investigated by culture-independent molecular tool. Table adapted from (Zhou, et al. 2007)	11
Table 3 - Sampling and location sites, sample type, type of land cover/land cover, geographic coordinates, sampling deep and date of collection.	16
Table 4 - OTU, organism, corresponding percentage similarity for the 16S rDNA bacterial partial sequences, NCBI taxonomy ID, NCBI reference sequence, Isolation source of the sample related, country/place, Pubmed ID of the paper and phylogenetic association.	36
Annex Table 1 -PCR-DGGE Bray-Curtis Similarity (group average) matrix data, REF – reference to each sample; Combining samples – The combination of different samples, based on their Ref, and the corresponding similarity.	52
Annex Table 2 - Pyrosequencing Bray-Curtis Similarity (group average) matrix data, REF – reference to each sample; Combining samples – The combination of different samples, based on their Ref, and the corresponding similarity.	53
Annex Table 3 - Resume of most abundant OTUs distribution for every sample, and respective phylogenetic association. (OTUs with > 50sequences)	54

1. Introduction

1.1. Dissertation Outline

The present dissertation begins with an introduction, addressing the scope, motivation, relevance and project aims, following a state of the art with a revision concerning the karst areas, present situation of the groundwater quality in Portugal, the study region, bacterial communities in aquifers (potential for pollution tracers), present molecular approaches to study bacterial communities, emphasising the importance of NGS nowadays.

A second part includes the description of materials and methods used in the research study, addressing the question of the thesis in aiming to evaluate the culture-independent DGGE as a screening approaches for assessing the bacterial diversity in samples, by comparing with pyrosequencing data.

The third part includes the Results and discussion from the determination, identification and characterization of the bacterial communities present in groundwater samples from Estremenho massif in Serra de Aire e Candeeiros, using molecular tools.

Finally, in the conclusion, the major outcomes from the study and future perspectives are also pointed out.

1.2. Scope, motivation and relevance

Water is the most precious natural resource for life on earth but extremely threatened by human activities, particularly when it comes to freshwater systems (Meybeck 2003). Agriculture and livestock, public water supply, industry (tanneries, mining, food processing, weaving, etc.) and tourism are some of the direct threats that drive changes in landscape structure, changing both biogeophysical and biogeochemical surface fluxes (Vörösmarty et al. 2010). In terms of the present worldwide land use/land cover, agriculture represents 11% of the total land area (Pielke et al. 2011), where 12,5% is estimated under irrigation (Biemans et al. 2011). Livestock industry represents 25% of the total land area and less than 5% is attributed to urban landscapes (Pielke et al. 2011). In short, over the past 300 years, this

represents a tremendous increase of land managed for human activities and population growth, leading to massive impacts on the environment and an increased demand for water resources in the near future (Biemans et al. 2011). In Portugal, agriculture represents near 43.8% (35% agriculture and 8.8% agroforest) of total land use/land cover, where 34.6% is attributed to forest area and 5% to industry and urban (LANDYN 2014).

Therefore, land uses/land cover and water resources are directly connected. A conclusion that is very important to consider, since about 97% of Earth's water is marine (in oceans and estuaries) and inappropriate to consume without any treatment, and only 3% is freshwater. From this 3 %, approximately 69% is locked up in the solid state (frozen water present in glaciers, ice and snow), 30% is groundwater and only 1% is present at the surface and distributed by rivers, lakes, wetland, etc. (Gleick 1993). This makes groundwater a fundamental natural source and reservoir of freshwater in the world, widely used for human consumption and socioeconomic development (Danielopol et al. 2003).

Worldwide, it is estimated an impressive use of 50% of groundwater as drinking water source, but in agriculture, this number can reach 70% for irrigation derived from groundwater (United Nations 2003).

In Portugal, the water used for human purposes is mostly for the agriculture sector (irrigation) and urban consumption, with groundwater representing 63% as a water source, as shown in Table 1 (Ribeiro and Cunha 2010).

Table 1 - Consumption values of surface and groundwater in Portugal. Adapted from (Ribeiro & Cunha, 2010)

	Surface Water		Groundwater	
	km ³ /yr.	%	km ³ /yr.	%
Irrigation	2.34	36	4.21	64
Urban	0.21	37	0.35	63
Industry	0.21	54	0.18	46
Tourism	0.01	50	0.01	50
Total	2.77	37	4.75	63

Groundwater is the cheapest and fastest way to fulfil human needs, making mandatory the need to protect these precious reservoirs nowadays and in the future, but also because of its largely known beneficial effect on the environment. Groundwater reservoirs have major roles in our environment, as they provide the base flow that maintains most rivers flowing all year long, influencing the character of aquatic ecosystems and enabling them to flourish (Lerner and Harris 2009); they are also intimately connected with the landscape that it underlies (Lerner and Harris 2009), which makes important the understanding of basic principles of interactions between groundwater and surface water for an effective management of water resources, as contamination of one commonly affects the other (Sophocleous 2002).

The natural movement of water that leaves the soil areas percolates through the unsaturated zone to the water table, carrying solutes (including anthropogenic pollutants) with it. The water and solutes then flow through the saturated zone until they eventually reach their discharge point (a spring, riverbed, seep or borehole) (Lerner and Harris 2009). Depending on the type of soil / rock, the discharge end may have a more adverse effect on groundwater, such as in the case of Karst areas. Worldwide, karst areas are characterised as a result of natural erosion of carbonate and evaporate rocks, in which the landforms are formed by sinking streams, natural bridges, dry valleys, enclosed depressions, caves, gorges, fluted rock outcrops, large springs, landforms produced by dissolving rock (Drew and Hötzl 1999). These characteristics make the karstic areas highly susceptible to anthropogenic contamination from surface points and diffuse sources of pollution.

On the other hand, the dynamics of exchange processes between surface water and groundwater are an important role for the structure of subterranean communities (Danielopol et al. 2003).

Both biotic and abiotic factors are essential to understand and study these environments. One of the most important biological parameters, includes the bacterial communities, as these play a critical role in the cycling of carbon, nitrogen and other elements, synthesising natural products, and impacting the surrounding organisms and environment (Stewart 2012). However, groundwater quality issues are almost exclusively addressed by abiotic/physicochemical criteria (Tomlinson et al. 2007; Lemarchand, Masson, and Brousseau

2004), leaving relevant biological data unstudied (Stein et al. 2010), especially in what concerns the bacterial communities (Axenov-Gibanov et al. 2016). Therefore, it becomes important to characterise their composition and diversity in these precious habitats. For instance, the simple quantification of chemicals in the environment is not enough to reveal the real effect of contamination on the aquifer, making necessary the assessment of the biological effects that pollution causes at different hierarchical levels, by considering all criteria for a correct evaluation. But to address important changes in the bacterial assemblages, it is necessary to build databases that can provide information for time-series research in a near future.

For Portuguese karst ecosystems, the information on bacterial composition and diversity is inexistent. Recent molecular approaches such as massive sequencing (Sun et al. 2012) have provided an important advance in understanding extreme environments and allow to study ecosystems where culturing approaches are not suitable. Therefore, for the first time, these molecular approaches were applied in the present work for the study of microbial communities from underground water at the Estremenho Karst Massif (Central-Western Portugal).

The present dissertation was developed within the scope of KARSTRISK project (PTDC/AAC-AMB/114781/2009), which aimed to assess the impact of anthropogenic activities on the groundwater ecosystems from the Estremenho Karst Massif and generate useful information for their protection, emphasising the need to achieve a good knowledge of biodiversity present in karstic areas, where biological communities are usually neglected.

1.3. Dissertation aims

Conducted within the scope of KARSTRISK project (PTDC/AAC-AMB/114781/2009), this study aimed to, for the first time, to identify and characterise the bacterial diversity and composition present in groundwater samples collected from springs, holes and boreholes from the Estremenho Massif, a karst area from Serra de Aire e Candeeiros (Central-Western Portugal). For this purpose, samples were examined using two different culture-independent

methods, the PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and Pyrosequencing. Potential similarities between the bacterial diversity of samples were explored in order to assess potential connections among the groundwater sources used in the study, evaluating also a possibility the use of bacterial communities as biological markers for groundwater from karstic ecosystems.

A second aim of the research was the evaluation of potential relationships between bacterial communities composition with pollution from surface anthropogenic activities at the Estremenho massif, for a future potential use as pollution bioindicators.

1.4. State of the Art

1.4.1. Karst areas and aquifers

Karst is defined as soluble rocks, the carbonate rocks (especially limestone, dolomite, and marble) and also evaporate rocks (gypsum and rock salt). Carbonate rocks outcrop across about 17.65 million square kilometres of the ice-free (excluding Antarctica, Greenland and Iceland) continental area of the Earth, representing about 13.2% of the world's land area, but this is just at surface, underground is considered that carbonate rocks involved in karst groundwater circulation extend karst area up to 16% of the world's land area (Williams 2008). In Europe (excluding Iceland and Russia), carbonate rocks outcrop across about 1.34 million square kilometres representing 21.8% of the Europe's land area (Williams 2008). In Figure 1 it's possible to see the distribution of all areas of carbonate rock outcrops in the world.

Karst landscapes are characterised by sinking streams, natural bridges, dry valleys, enclosed depressions, caves, gorges, fluted rock outcrops and large springs, impressive natural landforms produced by rainwater dissolving rock (also known as dissolution) (Ford and Williams 2007). But other natural processes can often interfere, such as glaciation or river erosion, which modifies the karst forms and produces intermediate landscape styles such as glaciokarst, fluviokarst, etc. (Williams 2008).

The intimate connection between surface and underground plays a vital role in the structure of subterranean communities due to the exchange processes between surface water and groundwater (Danielopol et al., 2003).

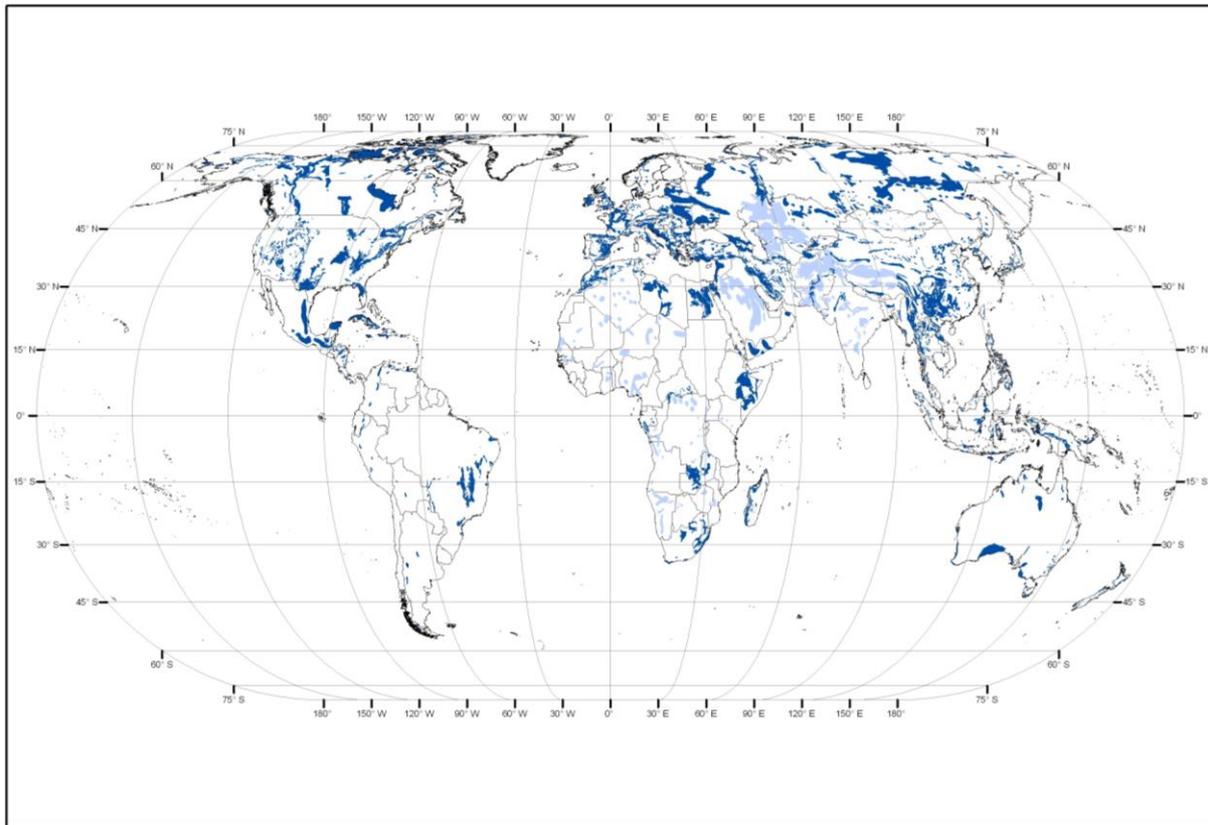


Figure 1 – Karst landscapes outcrops worldwide with subcrop mapped. Solid colour indicates that carbonates are relatively continuous; pale colour depicts areas in which carbonates are abundant but not continuous (adapted from Williams & Ford 2006).

In Portugal continental, 20% of the geographical extension is occupied by aquifers (62 aquifer system), asymmetrically distributed (APA 2015b) and 29 undifferentiated hydrogeological formations, in a total of 91 groundwater bodies distributed by eight different hydrographic regions (with exception of hydrographic regions from Madeira – RH9 and Azores – RH10) (INAG 2005). Three distended aquifers systems can be found in Portugal, the porous, karstic and fractured aquifers.

According to Almeida et al. (1995) the Portuguese carbonate rocks support important karst lands that occupy a considerable area in the Portuguese territory (Figure 3) (Almeida et al. 2000), including the major massif, the Estremenho massif (karst free aquifer) (Figure 4)

with 786 km² total area and 426,79 hm³/year of availability underground water annual (APA 2015a), which constitutes the study area of this thesis.

By law 58/2005, that transposes the Europe Union (EU) Framework Directive to the Portuguese legislation, ten hydrographical regions (RH) – see Figure 2, were established in Portugal for planning purposes: Minho and Lima (RH1); Cávado, Ave and Leça (RH2); Douro (RH3); Vouga, Mondego, Lis and Ribeiras do Oeste (RH4); Tejo (RH5); Sado e Mira (RH6); Guadiana (RH7); Ribeiras do Algarve (RH8) (APA 2015b). The protection of Groundwater against pollution, is regulated by the legislative framework directly associated with the implementation of the WFD (Water Framework Directive) and by the Decree-Law No. 208/2008 of 28 October transposed by directive No. 2006 Relative / 118 / EC, which establishes the protection of groundwater against pollution and deterioration (APA 2015b).

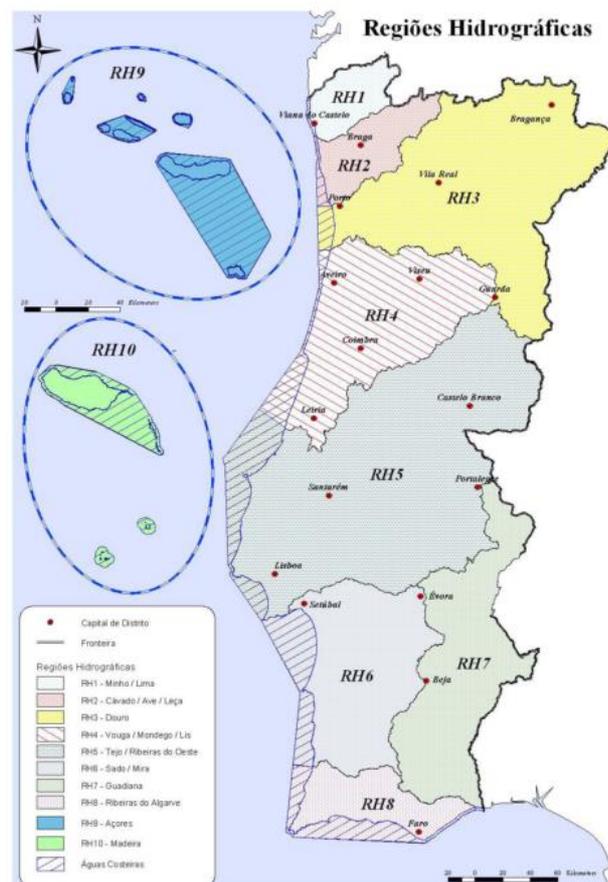


Figure 2 – Representation of Hydrographic regions in Portugal (adapted from INEG, 2005)

Even with all the legislation established, the classification of the water bodies is based on limited information due to the lack of monitoring of various important parameters and lack of representation and inadequate monitoring network in some of the regions for the targeted goals (APA 2015b).

According to the State of Environment Report from 2015, the chemical assessment of the state of groundwater bodies is positive at the national level, with problems of contamination of groundwater in some regions. For the quantitative evaluation of existing underground 91 water bodies, 29 % have a tendency to increase the groundwater level , 35 % reveal stability level , 19% indicate a downward trend the groundwater level and 17% could not perform trend analysis (APA 2015c).

The law defines criteria, to be used in assessing the chemical status of groundwater waterbodies (Arsenio, Cadmium, Lead, Mercury, etc.) but it doesn't define the criteria necessary to fully evaluate the biological communities that can be an important fraction for pollution evaluation.



Figure 3 - Major Karst areas (adapted from (Reboleira et al. 2011))

The Estremenho Massif is integrated into the national park of Serra Aire e Candeeiros (Central-Western Portugal), a protected area, created on May 4 of 1979 with an area of 38.900 hectares, which according to EU framework directive is part of RH4 and RH5 from Portugal (APA 2015a).

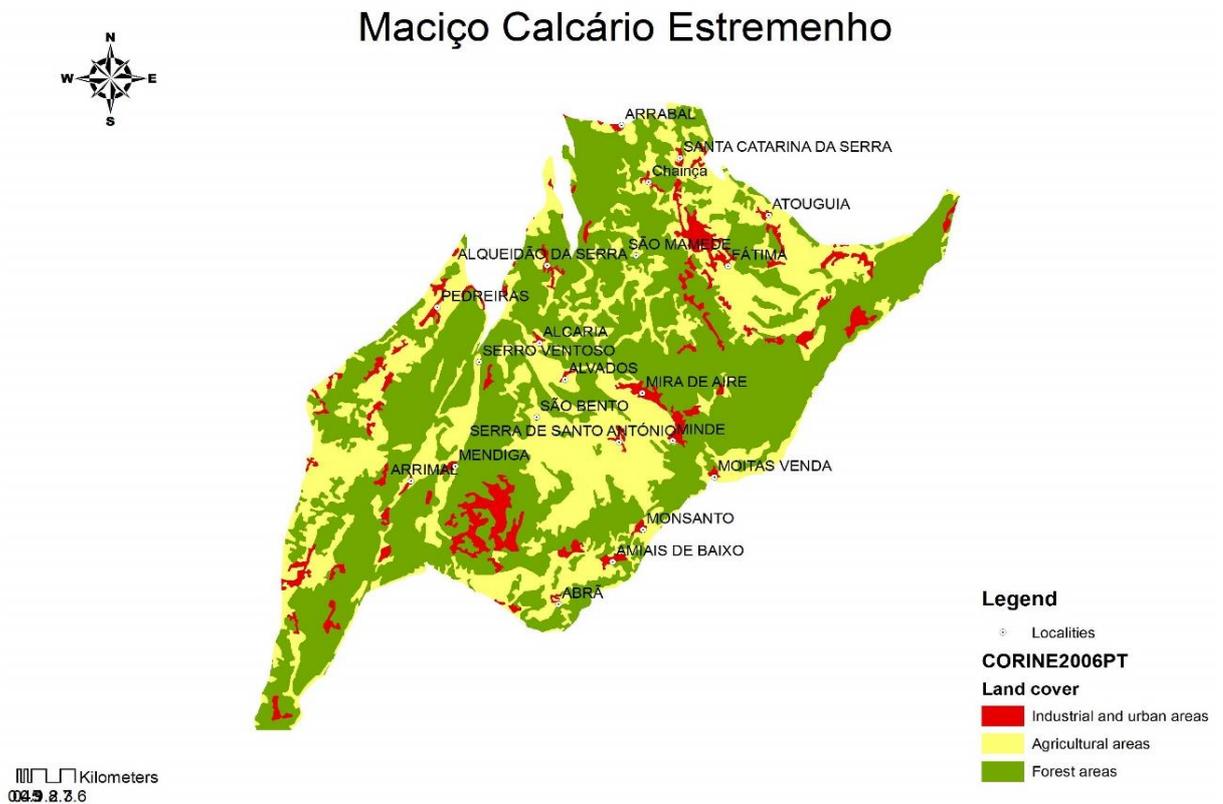


Figure 4 – Land use/land cover in Estremenho Karst Massif

The region presents a poor soil and water scarce on the surface (APA 2015a). For these reasons, the population and industry have to adapt to difficult natural conditions. Most of the industries can be found near karst springs with very high flow rates, which drain all the water that seeps into the Massif, increasing the pollution impact of aquifers. The modern industry is centred on stone exploitation, intensive pig farming and tanneries, while the traditional economy is based on olive oil production and cattle (ICNF 2015). All of these economic activities were part of economic history of this region without any regulation for decades, contributing to destruction and contamination of soil and habitat, scenic value of the

landscape change, decrease of air quality, aquifers contamination, abandoned waste and decrease of the fauna and flora of the area.

The tourism, in the last 30 years, has been also a major economic source in the region, most of it due to the presence of caves, putting at risk this protected areas.

1.4.2. Bacterial communities in groundwater from karst environments

Subterranean communities, like bacteria, have a critical role in the cycling of carbon, nitrogen and other elements (Iron, sulphur, etc.), synthesising natural products and impacting the surrounding organisms and environment (Stewart 2012). Regarding groundwater, the first study relating groundwater contamination with bacterial and virus associated with pit latrines was done in 1937 (Caldwell 1937). Until 1937, there was absolutely no concern regarding groundwater environment contamination. Fortunately, the evolution of methods for studying microbial diversity and the desire to better understand these environments led to an increase of knowledge and understanding of the role of microorganisms in the groundwater environment.

In 1982, Bruce H. Keswick and colleagues (Keswick, Wang, and Gerba 1982) proposed the use of microorganisms as groundwater tracers, an idea that raised after several poisoning outbreaks throughout history directly related to the result of poorly treated or untreated water (Ritter et al. 2002).

Therefore, it is mandatory to determine the presence or absence of microbial pathogens in water, especially in groundwater, as it represents the major source of freshwater in the world, as already described above. Ideally, monitoring for the presence of all pathogens should be made regularly, but this ideal situation is economically unsustainable. Instead, bioindicators microorganisms are employed for this purpose as an easier way to detect and enumerate the bioindicators microorganisms (Lemarchand, Masson, and Brousseau 2004). However, these may not represent the true impacts and changes in microbial communities. This is why the study of whole communities becomes an important direction for further research and monitoring strategies in the future (Goldscheider, Hunkeler, and Rossi 2006), particularly with the increasing use of molecular approaches and the decreasing costs

associated with them. However, there is much to study in these particularly environment and information is still scarce (Anantharaman et al. 2016)

Studies conducted worldwide in karst areas, investigated by culture-independent molecular methods analysing samples collected in caves, showed that bacterial composition (Table 2) is dominated by Proteobacteria in all studies reviewed, followed by Acidobacteria, Planctomycetes, Bacteroidetes, Chloroflexi, Nitrospirae, Actinobacteria, Firmicutes. A very interested fact is the similarity between sites so distant like present in two different continents: The Altamira Cave in Spain and Niu Cave in United States of America. This interesting fact can be explained by the strong possibility of these groups, especially the Proteobacteria phylum, being a major key in the role biogeochemical cycles in caves (Zhou et al. 2007).

Even without previous studies in Portugal (regarding bacterial communities) karst areas, a very identical bacterial diversity between karst areas can be expected during this study.

Table 2 - Distribution of bacteria in caves investigated by culture-independent molecular tool. Table adapted from (Zhou, et al. 2007)

Putative phylogenetic affiliation	% of clones							
	Nullarbor Caves (Holmes <i>et al.</i> , 2001)	Altamira Cave (Schabereiter-Gurtner <i>et al.</i> , 2002a)	Tito Bustillo Cave (Schabereiter-Gurtner <i>et al.</i> , 2002b)	Llonín Cave (Schabereiter-Gurtner <i>et al.</i> , 2003)	La Garma Cave (Schabereiter-Gurtner <i>et al.</i> , 2003)	Lechuguilla and Spider Caves (Northup <i>et al.</i> , 2003)	Lower Kane Cave (Engel <i>et al.</i> , 2004)	Niu Cave (this study)
<i>Proteobacteria</i>	40.0	52.3	48.8	59.3	32.8	35.0	92.7	42.6
<i>Acidobacteria</i>		23.8	29.2		24.1		5.6	18.6
<i>Planctomycetes</i>	5.7	4.8	2.4					9.0
<i>Bacteroidetes</i>	8.6	9.5	2.4	11.1	3.5		1.7	2.1
<i>Chloroflexi</i>		4.8	7.3		1.7			7.5
<i>Nitrospirae</i>	5.7			3.7	3.5	15.5		8.0
<i>Actinobacteria</i>	5.7	4.8	9.8	22.2	19.0	11.7		6.4
<i>Firmicutes</i>				3.7	13.8	37.9		
<i>Gemmatimonadetes</i>								2.7

1.4.3. Metagenomics approaches for the study of microbial communities

Metagenomics applies a suite of genomic technologies and bioinformatics that can be used to analyse entire microorganism communities by using genetic material collected directly from the environment samples (water, soil, etc.), providing a survey of most microorganisms present, without traditional detection methods based on the use of selective

culture, standard biochemical methods or taxonomic analysis (Handelsman 2004). This brings plenty of advantages for bacterial studies, as only a small fraction of the total diversity (>99%) that exists in nature can be cultured *in vitro* (Stewart 2012; Hugenholtz, Goebel, and Pace 1998).

Experimentally, a typical metagenomics project passes through several steps: experimental design, the sampling processing, sequencing technology, assembly, binning, annotation, statistical analysis and data storage and sharing (Thomas, Gilbert, and Meyer 2012).

Different molecular techniques, analysis software and sources of DNA have been used in metagenomic studies during years. Most of the studies were focused in the relatively short sequences, generally conserved within a species, and often different between bacterial species, the 16S ribosomal RNA sequences (Ellegaard and Engel 2016). However, the fast and substantial cost reduction in next generation sequencing (NGS) has exponentially accelerated the development of sequence-based metagenomics (Thomas, Gilbert, and Meyer 2012; Techtmann and Hazen 2016). The field of metagenomics is still responsible for considerable advances in microbial ecology, evolution, and diversity by allowing to understand the uncultured majority of microbial life.

Pyrosequencing is one of the results of evolution in NGS, which has been used at large scale in metagenomics worldwide and it is still in evolution leading to the rise of new technologies, such as the Ion torrent technology, developed by Thermofisher scientific (Ansorge 2015). Pyrosequencing was developed in 1986, when Pal Nyrén had a simple idea for an alternative DNA sequencing technique to the Sanger method, although the technique was fully developed at the Royal Institute of Technology in Stockholm in 1996 by Mostafa Ronaghi and Pål Nyrén (Metzker 2007). The basic concept of pyrosequencing is to follow the activity of DNA polymerase during nucleotide incorporation into a DNA strand, by analysing the pyrophosphate (PPi) released during the process by proportionally converting it into visible light using a series of enzymatic reactions (Metzker 2007). These enzymatic reactions are monitorised by bioluminescence, which occurs when reactions between PPi and Adenosine-5'-phosphosulfate (APS), releasing ATP. The ATP acts as a substrate for the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in

proportional amounts to the amount of ATP (Ehn et al. 2004). The light produced in the luciferase-catalysed reaction is detected by a camera and analysed in a program.

The first next-generation system on the market was Genome Sequencer (GS) instrument developed by 454 Life Science in 2005 (Margulies et al. 2005). In this system, the whole genome could be sequenced without any cloning step, after random shearing, specific adaptors would be ligated to the two ends of the DNA fragment, allowing the DNA molecules captured on the surface of a bead to be amplified by emulsion PCRs and isolated within an emulsion droplet. The aim of emulsion PCR is to get enough light signal intensity for reliable detection. The major drawback of GS is the relatively high error rates come from misjudging the length of homopolymer runs result in single base insertions or deletions. The relatively high cost of operation and generally lower throughput are the main limitations of the method (Life Science 2014), but the information output generated from sequencing is far better than the traditional cultured-based methods or previous culture-independent techniques such as Denaturing Gradient Gel Electrophoresis (DGGE).

The pyrosequencing technique brought an innovative vision to study more effectively the genomics of the microorganisms, and for years, it was one of the greatest contributor technique, to metagenomics. However, new technologies with different principles, from pyrosequencing rise every day contributing for a better, faster and cheapest away to sequence genetic material. For instance, Illumina platform provides a high throughput at a smaller and more modestly priced platform and the nanopore sequence technique (Oxford nanopore platform), single-molecule real-time (SMRT) technology (Pacific Biosystems) (Ansorge 2015).

Nowadays, the biggest challenge in this area is the management of large amounts of information. In the case of metagenomics, it's inevitable the automation of the analysis process in the future, in a more efficient and less time-consuming manner, making the analysis that currently takes days/weeks in a few hours. But for now lot of effort continues to be needed to analyse data from microbial diversity, using software such as MLST (<http://www.mlst.net/>); MOTHUR (<http://www.mothur.org/>); EstimateS (<http://viceroy.eeb.uconn.edu/EstimateS/>); PHACCS (<http://phaccs.sourceforge.net/>); or QIIME (<http://qiime.org/>) (Schloss et al. 2009; Caporaso et al. 2010; Angly et al. 2005).

QIIME (Quantitative Insights Into Microbial Ecology) was the open-source software pipeline selected to analyse microbial diversity in this study, that processes data from a high-throughput 16S rRNA sequencing (such as pyrosequencing) and performs microbial community analysis, solving the problem of taking sequencing data from raw sequences to interpretation and database deposition (Caporaso et al. 2010). It can be used to analyse and interpret nucleic acid sequence data from bacteria, fungal, viral and archaeal communities (Kuczynski et al. 2011).

The analyses consist of typing a series of commands into a terminal (Linux OS), generating textual and graphical output data (Kuczynski et al. 2011) using the raw data generated in the Roche/454 platform. Three files are generated, FASTA file (.fna), that contains the sequence converted from the original flowgrams (light signal strength), the Quality Scores (.qual), which contains a score for each base in each sequence include in the FASTA file, and finally the Mapping File (.txt), that holds all samples information necessary to perform data analyses (Kuczynski et al. 2011). In the next step, the samples are assigned to the multiplex reads based on barcodes, performing a quality filtering removing low-quality and ambiguous reads. At this stage the most frequently used practice for organizing fine scale bacterial diversity is to cluster sequences exclusively on the basis of DNA sequence similarity at a conserved locus (Koeppel and Wu 2013), the cluster sequences called Operational Taxonomic Units (OTUs), allowing picking a representative sequences from each OTU-based on their sequence similarity. By default, QIIME, the sequences are clustered at 97% sequence similarity (Kuczynski et al. 2011), resulting cluster typically thought of as representing a biological species.

Several studies using metagenomics for the study of groundwater environments have been published (Moss, Nocker, and Snyder 2011; Hemme et al. 2015; Zhou et al. 2007; Schabereiter-Gurtner et al. 2002a; Schabereiter-Gurtner et al. 2002b; Schabereiter-Gurtner et al. 2004), highlighting the usefulness of this approach to characterize environments where most organisms from microbial communities are not possible to grow in laboratory cultures making molecular culture-independent methodologies very import for metagenomic studies. For Portugal, much information on bacteria from groundwater ecosystems is still inexistent, which makes the present study an important first screen basis.

2. Materials and Methods

2.1. Sampling

A set of 22 groundwater samples were collected from boreholes, wells, a river and water springs from the Estremenho Karst Massif, located in Central-Western Portugal.

As described above, the Estremenho Massif belongs to the national park of Serra Aire e Candeeiros (Central-Western Portugal), a protected area of 38.900 hectares and it is the major karst massif in Portugal with a karst free aquifer of 786 Km² with an underground water availability of 426.79 hm³/year (APA 2015a). There are several pressures related to potential pollution from industrial and agriculture activities. The location name of sampling sites, the different water volumes collected, also the land cover/land cover, geographic coordinates, sampling date and deep of collection (not always possible to measure), all of this data was synthesised in Table 3. The sampling sites are marked on the map in Figure 5.

This sampling campaign was conducted in February 2013.

2.2. DNA extraction

Total DNA from environmental water samples was extracted as follows: water samples (volume ranging from 500 to 5000mL, depending on the filter clogging) were filtered through 0.20µm polycarbonate sterile filters, resuspended in 2 ml of TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0] and centrifuged; lysozyme was added after resuspension in 200µl of TE buffer and incubation was performed at 37°C for 1 h; the following DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania); 600 µL of chloroform were added and mixed by inversion and centrifuged at the highest velocity for 10 minutes; the aqueous phase was transferred to new tube and 1 volume of isopropanol was added, mixed by inversion and incubated at 4°C for 30 minutes, following centrifugation at the highest speed for 30 minutes; the supernatant was discarded and

Table 3 - Sampling and location sites, sample type, type of land cover/land cover, geographic coordinates, sampling deep and date of collection.

Site	Location	Sample type	Land cover/land use	Lisboa Hayford gauss lgeoe		Deep (m)	Sampling Date
				M X	PY		
K01	Alviela Spring	Water spring	Forest area	150043,00	275614,00	34	27FB12
K02	Amiais riverside	River	Forest area	150166,02	276674,22	22	27FB12
K03	Almonda Spring	Water spring	Forest area	158424,00	282140,00	12	27FB12
K04	Nascente do Lena	Water spring	Forest area	140961,00	290852,00	24	18FB12
K06	Fountain – Lis spring	Water spring	Forest area	145277,58	302236,27	30	18FB12
K07	Well da Ribeira do Lena	Well	Agricultural area	140969,26	290865,96	19	18FB12
K08	Fountain Alqueidão da Serra	Bore	Forest area	143702,33	294962,89	15	18FB12
K09	Chiqueda	Bore	Forest area	129725,28	286042,20	32	19FB12
K10	Moleanos	Bore	Forest area	132786,05	283365,49	-	19FB12
K11	Lagoa do Furadouro	Bore	Industrial and urban area	163802,41	294925,68	120	27FB12
K13	Giesteira	Bore	Forest area	153897,27	292004,6	169	27FB12
K15	Fátima	Bore	Industrial and urban area	155331,69	294570,35	277	27FB12
K16	Fátima	Bore	Agricultural area	152362,52	296468,00	400	18FB12
K18	Valverde - Alcanede	Bore	Industrial and urban area	138293,22	275809,88	350	20FB12
K19	Valverde - Alcanede	Bore	Industrial and urban area	138286,06	276022,14	114	20FB12
K20	Serra de Stº António	Bore	Agricultural area	149536,33	282273,46	250	20FB12
K21	Covão do Feto	Bore	Agricultural area	151533,56	280899,31	-	20FB12
K22	Covão do Coelho	Bore	Forest area	153962,90	284350,89	404	20FB12
K23	Tremoceira - Pedreiras	Bore	Agricultural area	137234,47	293210,57	200	19FB12
K24	Casais de Santa Teresa	Bore	Industrial and urban area	135647,01	289282,98	220	19FB12
K25	Moita well	Bore	Forest area	129700,86	276314,14	-	18FB12
K30	-	Bore	Forest area	129962,6	285784,4	-	19FB12

resuspension of pellet was made in 100 µL of NaCl 1.2M; 2,5 volumes of absolute ethanol at -20°C were added and mixed by inverting, incubating at -20°C for 30 min and centrifugation at the highest speed for 30 min; the supernatant was discarded and the pellet was washed with 200 µL ethanol 70%; the pellet was dried and resuspended in TE buffer and stored at -20°C.

2.3. PCR-DGGE

2.3.1. PCR amplification of bacterial 16S rDNA fragments

Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA gene fragments was performed with the primers 338F-GC (5'-actcctacgggaggcagcag-3') and 518R (5'-attaccgctgctgctgg-3') (Muyzer et al. 1993). Primers were synthesised by STABVida (Oeiras, Portugal). PCRs were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) with a 35µl reaction volume with DreamTaq DNA Polymerase (Thermo Scientific) DNA polymerase. The PCR program had an initial denaturation step at 94°C for 5 minutes followed by 30 cycles of 30 seconds at 92°C, 30 seconds at 55°C and 30 seconds at 72°C, and a final extension step at 72°C for 30 minutes. Negative control reactions without any template DNA were performed simultaneously and the size of amplicons was verified by electrophoresis in a 1.5% agarose gel (SeaKem LE Agarose) using 100bp Plus DNA ladder as molecular weight marker (Vivan Technologies) and RedSafe Nucleic Acid staining solution by iNtRON biotechnology. The gel was visualised on an Ultra Violet (UV) transilluminator

In order to solve the low amplification in some samples, a nested-PCR was performed, using the same pair of primers and the PCR amplicon from the first sequencing as DNA template.

2.3.2. Denaturing gradient gel electrophoresis (DGGE)

PCR products were analysed through DGGE using a 35–60% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1 mm vertical polyacrylamide gels (8% [wt/vol] acrylamide in 0.5× TAE buffer). Electrophoresis was

performed in a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, California, USA) using 1× TAE buffer containing 40 mM Tris, 20 mM acetic acid and 1 mM EDTA (pH 8.0) during 17 hours and 30 minutes at 75 V. The gel was then stained for 5 minutes in an ethidium bromide solution (5%) and visualized on a UV transilluminator.

Image digitalization was performed using a Molecular Imager FX™ system (Bio-Rad Laboratories, Hercules, California, USA). For image analysis, the BIO-RAD software was used to determine correspondent bands and relative intensity of each band per lane. Cluster analysis of bands distribution was performed using the unweighted pair group mean average (UPGM) method based on the Bray-Curtis similarity coefficient and Metric Multidimensional scaling (MDS) with PRIMER 6 software (Clarke & Gorley, 2006).

2.4. Pyrosequencing

2.4.1. Sequencing

Samples showing a high yield in PCR amplification were chosen for pyrosequencing. Pyrosequencing libraries were obtained using the 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, UK). This allows the sequencing of a whole genome without any cloning step (Margulies et al. 2005). After random shearing, specific adaptors will be ligated to the two ends of the DNA fragment, allowing the DNA molecules captured on the surface of a bead to be amplified by emulsion PCRs and isolated within an emulsion droplet. The aim of emulsion PCR is to get enough light signal intensity for a reliable detection.

In the present study, bacterial 16S rRNA gene fragments from the V3V4 hypervariable region were amplified using barcoded fusion primers with the Roche-454 Titanium sequencing adapters A and B (an eight-base barcode sequence), the forward primer 5'–ACTCCTACGGGAGGCAG-3' (338F) and the reverse primer 5'–TACNVRRGTHCTAATYC -3' (802R) (Wang and Qian, 2009). PCR reactions were amplified in 20 µL reactions with Advantage Taq (Clontech) using 0.2 M of each primer, 0.2 mM dNTPs, 1X of polymerase mix, 6% DMSO and 1-2 µL of template DNA. The PCR conditions were 94°C for 4 minutes, followed by 25 cycles of 94°C for 30 seconds, 44°C for 45 seconds and 68°C for 60 seconds and a final

elongation step at 68°C for 10 minutes. The amplicons were quantified by fluorometry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA) at Biocant (Cantanhede, Portugal).

2.4.2. Sequences analysis using QIIME

Barcoded pyrosequencing libraries were analysed and processed using QIIME package software (Caporaso et al. 2010) in a virtual machine, Oracle VM VirtualBox (Version 5.0.22 r108108). The QIIME analysis consists of typing a series of commands into a terminal (Linux OS), generating textual and graphical output data (Kuczynski et al. 2011) using for microbiome analysis from raw DNA sequencing data. The analyses was performed as previously described in (Cleary et al. 2015). QIIME, fasta and qual files were used as input for the `split_libraries.py` script, which contains a score for each base in each sequence include in the FASTA file and finally the Mapping File (.txt), that holds all samples information necessary to perform data analyses (Kuczynski et al. 2011). Backward primers were removed using the 'truncate only' argument and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. In addition to user-defined cut offs, the `split_libraries.py` script performs several quality filtering steps (http://qiime.org/scripts/split_libraries.html). OTUs were selected using UPARSE with `usearch7` (Edgar 2013). The UPARSE sequence analysis tool (Edgar 2013) provides clustering, chimera checking and quality filtering on de-multiplexed sequences. Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera checking algorithm currently available (Edgar et al. 2011). The quality filtering as implemented in `usearch7` filters noisy reads and preliminary results suggest it gives results comparable to other denoisers such as `AmpliconNoise`, but is much less computationally expensive (<http://drive5.com/usearch/features.html>; last checked 2016-10-05). First reads were filtered with the `-fastq_filter` command and the following arguments `-fastq_truncflen 250 -fastq_maxee 0.5 -fastq_truncqual 15`. Sequences were then dereplicated and sorted using the `-derep_fulllength` and `sortbysize` commands. OTU clustering was performed using the -

cluster_otus command. An additional chimera check was subsequently applied using the uchime_ref command with the gold.fa database (<http://drive5.com/uchime/gold.fa>). AWK scripts were then used to convert the OTUs to QIIME format. In QIIME, representative sequences were selected using the pick_rep_set.py script in QIIME using the 'most_abundant' method. Reference sequences of OTUs were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the rdp method (Wang et al. 2007). In the assign_taxonomy.py function, we used a fasta file containing reference sequences from the Greengenes 13_5 release for and the rdp classifier method. We used a modified version of the taxonomy file supplied with the Greengenes 13_5 release to map sequences to the assigned taxonomy. Finally, we used the make_otu_table.py script in QIIME to generate a square matrix of OTUs x samples.

Closely related organisms of numerically abundant OTUs were identified using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'blastn' tool with the -db argument set to nt (Z. Zhang et al. 2000).

2.4.3. Statistical analyses

The rarefaction curve was determined using PAST software (Hammer et al. 2001). Cluster analysis of OTUs was performed using the unweighted pair group mean average (UPGM) method based on the Bray-Curtis similarity coefficient and Metric Multidimensional scaling (MDS) with PRIMER 6 software (Clarke & Gorley, 2006).

3. Results and Discussion

3.1. PCR

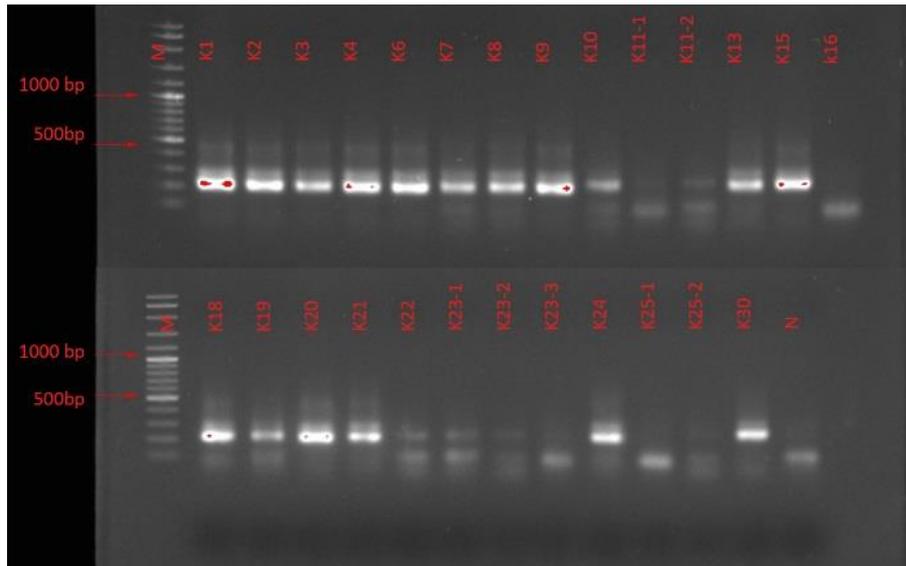


Figure 6 – Agarose gel (1,5%) electrophoresis of PCR products; Samples K1 to K30; Molecular size marker is marked with the letter M the Negative with the letter N

A nested-PCR was performed for all samples due to the initial low yield of amplification. After this nested-PCR (Figure 6) using the same primers 338F-GC and 518R, the gel showed the existence of the anticipated target fragments for samples K01, K02, K03, K04, K06, K07, K08, K09, K10, K13, K15, K18, K19, K20, K21, K24, K30. For samples K10, K11-1, K11-2, K16, K22, K23-1, K23-2, K23-3, K25-1, K25-2 there were very weak or lacking bands corresponding to the desired region (± 200 bp). Samples K11, K23 and K25 were divided into sub-samples, during filtering, because of the presence of debris/soil that clogged very quickly the $0.20\mu\text{m}$ holes of the polycarbonate sterile filters.

The low or no amplification of this specific samples, lead to assume three plausible ideas. First, the possibility of low or completely absence of bacteria DNA in this samples amplified. Second, in the samples K11, K23 and K25, it was clear the presence of soil/debris in the water collected, once the water was turbid what made very complicated to filter the water, resulting in a low volume filtered, for these samples, but not explaining the absence/low content of DNA in the samples K10, K16, K22 which were not subjected to a sub-division during filtration. Thirdly, the presence of PCR inhibitors, a group of substances, with different properties and mechanisms of action, which interact with DNA template and the polymerase, preventing the

enzymatic reaction (Wilson 1997). Substances like debris, fulmic acids and humic acids while described, predominantly found in water/environment samples (Abbazadegan et al. 1993; Marian Ijzerman, Dahling, and Shay Fout 1997). These questions should be addressed in the future in sampling design and DNA extraction from similar water samples.

3.2. 16S rDNA-DGGE band patterns and cluster analysis

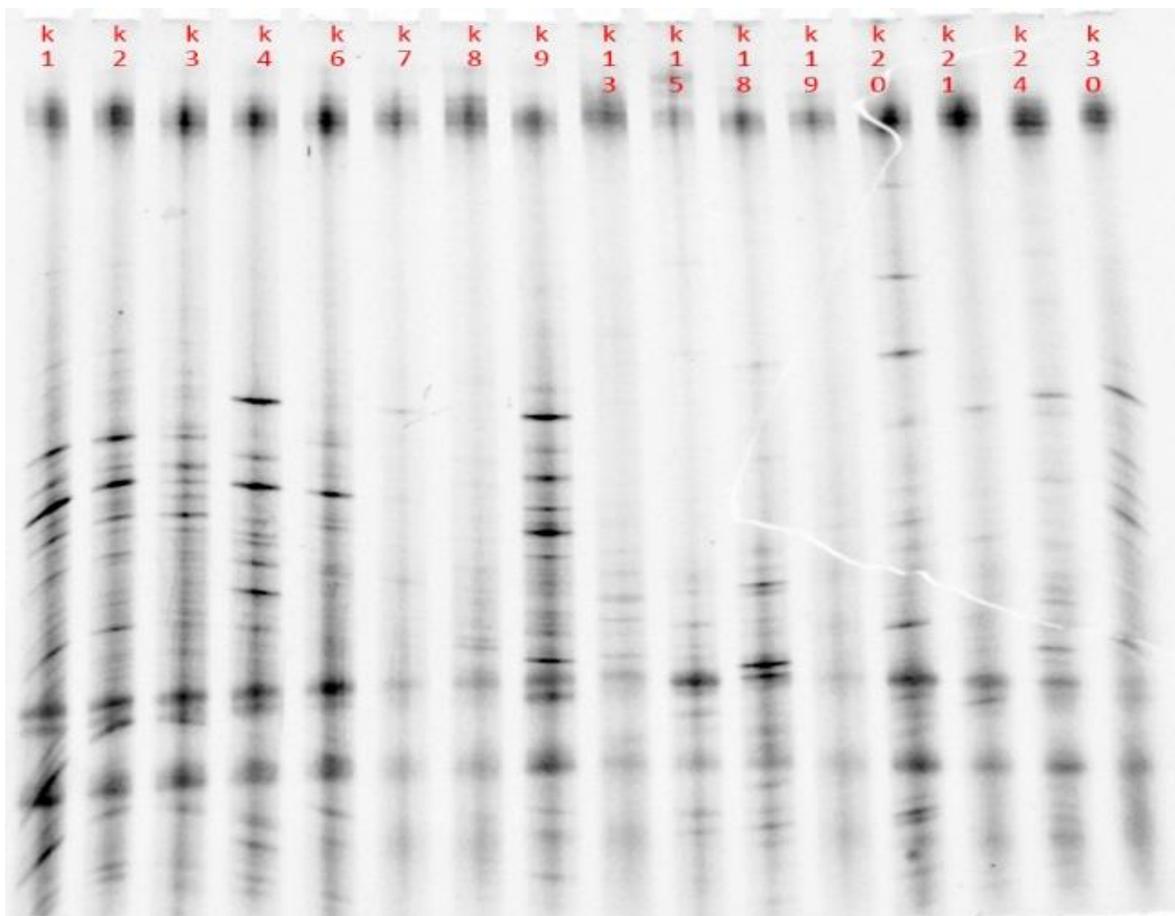


Figure 7 - PCR-DGGE profiles containing the bacterial 16S rDNA fragments from water samples k1, k2, k3, k4, k6, k8, k9, k13, k15, k18, k19, k20, k21, k24, k30 collected at Estremenho Kast Massif during February 2012

For DGGE analysis, a gel covering a total of 16 samples was carried out with the amplicons from the previous PCR using primers 338F_CG/518R (Figure 7). This resulted in a complex DGGE profiles from which a total of 228 bands could be detected, corresponding to 42 different band migration positions. DGGE band patterns were very complex to interpret

although, for samples K1, K2, K4, K6 and K9, DGGE profiles could be more clear, having visible common peaks and clearly there were several DGGE dominant bands that could be detected across samples' profiles. However, there were many weak bands corresponding to individual bacterial identities that resulted in a smear. For samples K7, K8, K13, K15, K19; K21 and K24, the profiles were not so clear and dominant bands were not so evident. These constrain made difficult to make bands correspondence analysis. This is one of the problems with DGGE band profiles analysis. There is also the fact that DGGE is based on the assumption that all samples have the same DNA content for PCR amplification, which is definitely not the case in these samples, where even the volume of filtration depended on several factors such as the suspended solids that rapidly clogged the filters. In fact, for some samples, the filter did not even clog after filtering 5L of water sample. This is a problem related to water samples from pristine water environments, where the abundance of organisms is very low (Goldscheider, Hunkeler, and Rossi 2006) and results in a low DNA extraction. Due to these gel analysis constraints, only most dominant bands (above 0.5% relative intensity in each lane) were considered for cluster analysis (using the Bray-Curtis similarity coefficient) – see Figure 8 and data matrix in Annex Table 1.

On the other hand, in spite of the bands/peaks and migration positions from DGGE gel profiles can correspond to individual microbial identities and the band intensities to their relative quantity (T. Zhang and Fang 2000), this does not mean that it is always valid to assume that one band refers to a specific genome, they are an indicative approach only. Moreover, two sequences that differ only by 2 base pairs may show indistinguishable band migration points in DGGE gels, which highlights one more limitation of using this technique (Jackson et al. 2000).

Besides the common disadvantages associated to traditional DNA-based techniques (DNA extraction efficiency; PCR errors; contaminations introduction, etc. (Muyzer 1999) another big disadvantage is associated to this technique, the difficulty of obtaining the bacterial identity correspondence to each band. This problem can be surpassed through NGS, approaches, making possible a more accurate association to specific bacteria, avoiding the time-consuming bands excision, cloning and Sanger sequencing procedures.

Nevertheless, DGGE has shown to be a valuable screening technique for assessing and compare the biodiversity among samples in a more affordable way and it is still used as a basis for further NGS analysis (Braun et al. 2016; Cleary et al. 2012).

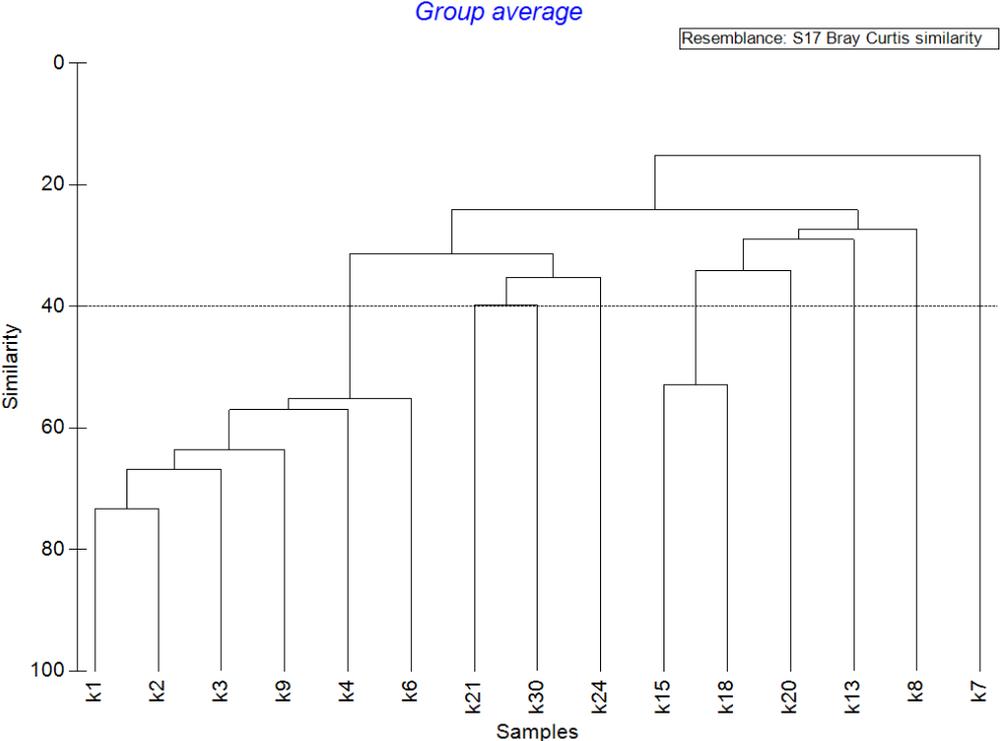


Figure 8 – Bray-Curtis similarity. Cluster dendrogram, with slicing at 40% similarity.

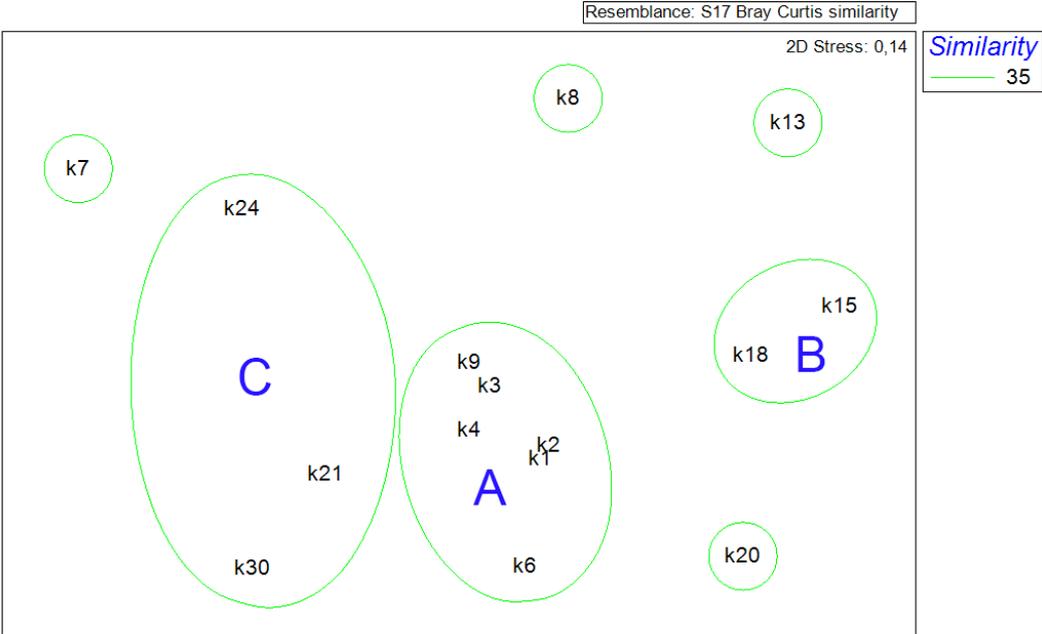


Figure 9 – Metric Multidimensional scaling (MDS) with resemblance level of 35%. Formation of 3 groups A, B and C.

After the results from Cluster analysis, it was clear that samples K1 and K2 showed the highest similarity, with 73.27%. This is not surprising, as these samples are very close geographically and share the same water origin (K2 rises to surface from K1). At this Massif, rivers are not perennial. Instead, they are temporary streams that arise to surface from springs (Rodrigues and Fonseca 2010), which explains the almost identical bacterial composition between K1 and K2. However, K3 also showed an important percentage of similarity (66.84%) with both these samples, in spite of its relatively distant location. This may somehow suggest an underground connection between samples K1 and K3, which are both water springs, and considering the high complexity of galleries and conduits in the massif (Rodrigues and Fonseca 2010; Hoffmann et al. 2013) this would not be such a surprise. Contrarily, K7 was the sample with less similarity (15.19%) with all the other samples; it is a well with a low depth, which can be easily impacted by surface pollution pressures that can dramatically influence the bacterial communities present in it. However, geochemical analyses (unpublished data) did not show dramatic different environmental variables in relation to samples K1 to K3, except for a higher value for ORP (oxidation reduction potential), which is a parameter reported to have an influence on the distribution of particular bacteria (Lu, Wilson, and Kampbell 2006).

At 35% similarity it was visible, the formation of three groups by MDS analysis (Figure 9), A (K01, K02, K03, K04, K06, K09), B (K15, K18) and C (K21, K24, K30). In these cases, the land use of the surrounding areas may also have a relevant impact on the specific bacterial communities' composition, as group A is exclusively surrounded by forest areas and B and C groups by agricultural and industrial/urban areas.

However, group A samples showed faecal contamination (unpublished data) which indicates an impact from surface anthropogenic pollution and will surely have a major role in the composition and diversity of their bacterial assemblages (Hemme et al. 2015; Anantharaman et al. 2016). On fact, most of these samples were collected at surface and the impact should be expected due to the direct interactions with surface environments. Group B samples were collected in boreholes and they are characterised by pristine environments but very high conductivity values, near 2000 $\mu\text{S cm}^{-1}$ (unpublished data), which is also characteristic from deep groundwater samples. Samples from group C were also collected from boreholes and are characterised by moderate conductivity and also no faecal

contamination (unpublished data). Therefore, there are many environmental variables that can influence the bacterial assemblages in groundwater ecosystems, which changes its diversity in order to respond to the new biogeochemical contexts that arise from the pollutants impact and dramatically changing the bacterial communities present (Hemme et al. 2015).

3.3. Pyrosequencing

Based on the first screening of bacterial diversity and intensity of band profiles provided by PCR-DGGE results (diversity and intensity of profiles) it was decided to send for sequencing only 13 samples: K01, K02, K03, K04, K06, K07, K08, K09, K15, K18, K19, K20, K24.

Cluster analysis of Sequence data

From the cluster (Bray-Curtis Similarity) and MDS analysis of pyrosequencing data (Figure 10, Figure 11 and Annex Table 2), it was clear the formation of a major group A (including samples K01, K02, K03, K04, K06 and K09) at 35% of similarity. This clustering of samples was already suggested by the cluster analysis using PCR-DGGE data (Figure 8), showing a strong connection between samples K01, K02, K03, K04 and K09. However, with pyrosequencing data, there was no establishment of other groups, as suggested by PCR-DGGE profiles, with sample K15 completely isolated from the other samples (only with 0.78% similarity).

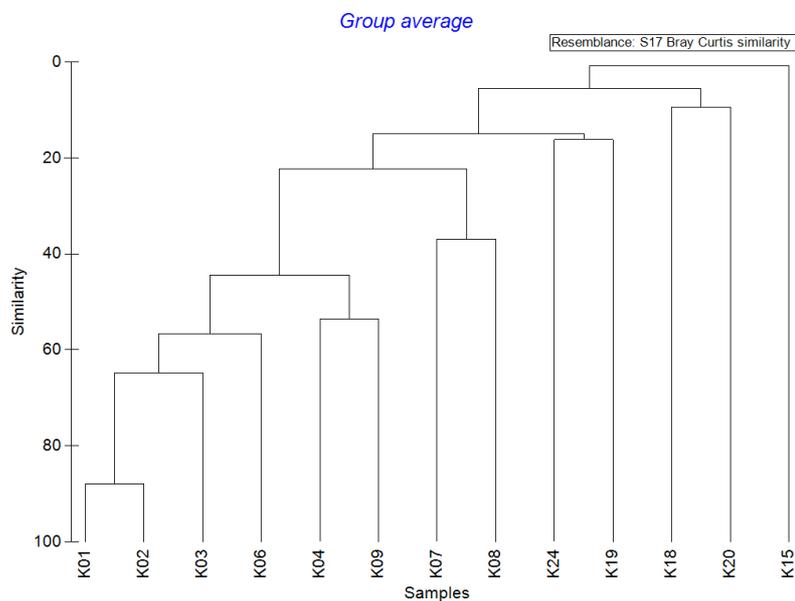


Figure 10 –Cluster showing OTU similarity between site samples (relevance >1%) with Bray-Curtis distance.

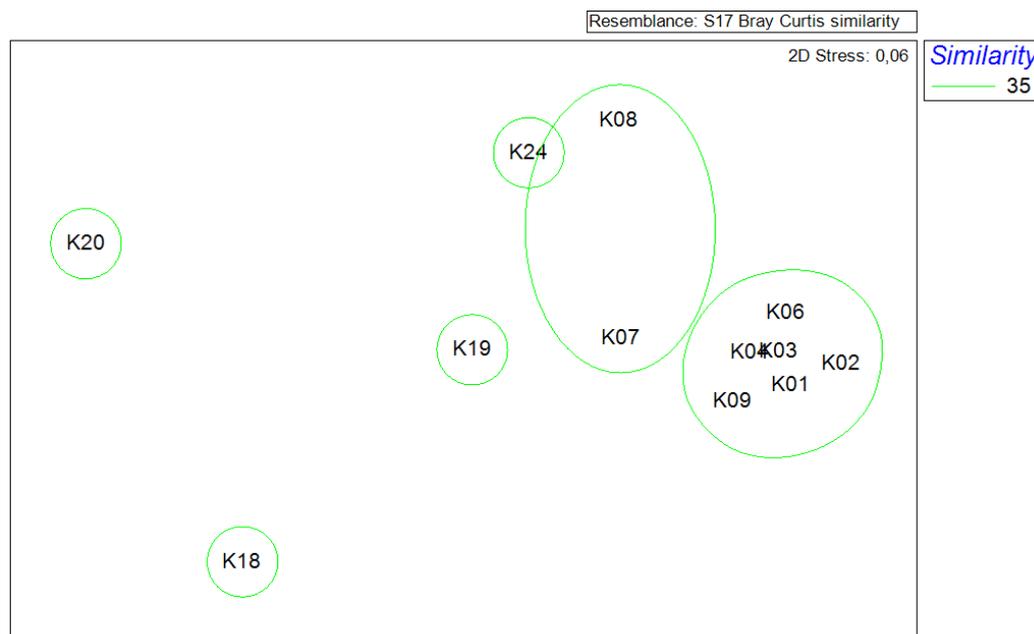


Figure 11 - Metric Multidimensional scaling (MDS) with resemblance level of 35%. The sample K15 was excluded.

OTU richness

The rarefaction curves of the 13 samples sequenced, as shown in Figure 12, provide the OTU richness for each sample, given a sampling depth (i.e. number of individuals/sequences per sample). Samples K01, K02, K04, K06, K09, K20 showed a medium OTU richness with a low number of sequences per OTU. By comparing to DGGE results, these samples showed, in fact, some few clear dominant bands (smear bands) in corresponding profiles showing a consistency in diversity across both molecular approaches. Samples K03, K24, K18, reveal also a medium OTU richness, but more sequences attributed to each OTU. Noticing that sample k15 showed the lowest OTU richness and the highest number of sequences (1592) suggesting the presence of one or very few bacterial groups, data supported by DGGE where actually showed the dominance of one main band. On the other hand, it was evident the highest richness was observed for samples K19 and k07, with 603 OTUs for 1573 sequences reads and 481 OTUs and 1142 sequences reads, respectively, both samples having the lowest concentration of sequences (less than 50 sequences) per OTUs. In DGGE gel image, this was also visible, with no major dominant bands in their profiles.

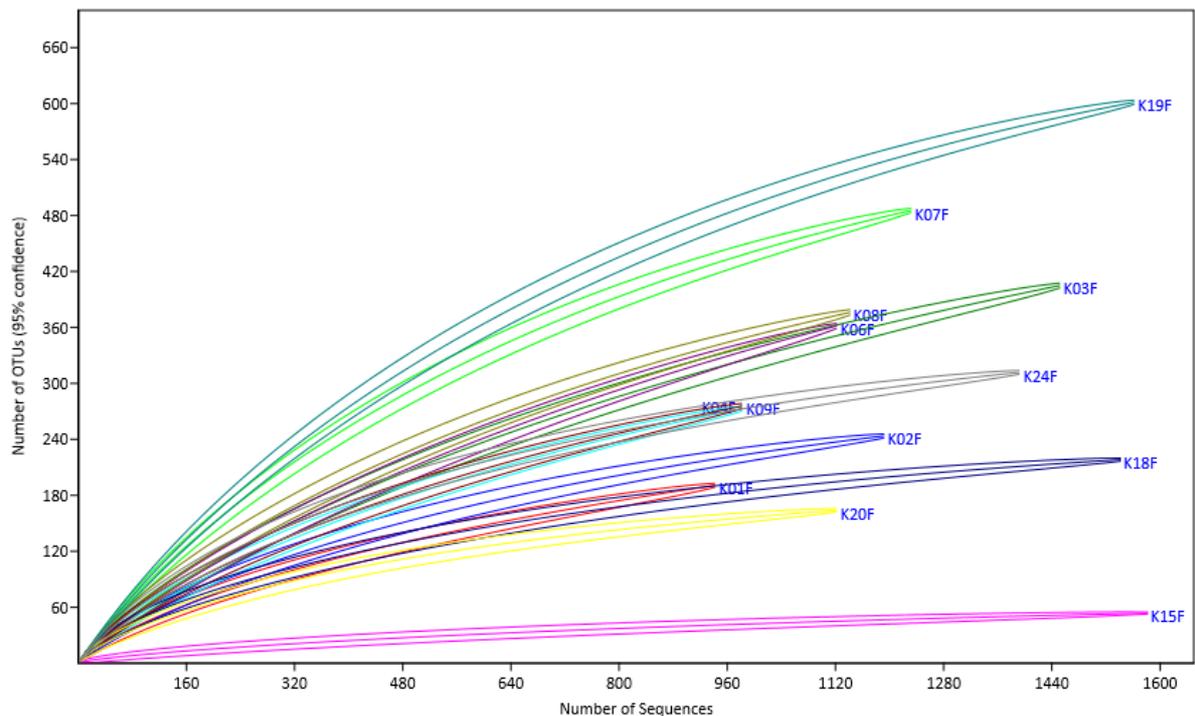


Figure 12 - Rarefaction curves of the 13 samples. The error bars show 95% confidence of upper and lower limits

Compositional sequence analysis

Barcoded pyrosequencing analysis yielded 16426 sequences, which were assigned to 1729 OTUs after quality control, OTU picking and removal of chimaera, sequences not assigned to the Bacteria domain and chloroplasts.

Those OTUs were assigned to 32 phyla, of which Proteobacteria (61.83%, 1069 OTUs) was by far the most abundant phylum in terms of sequences and OTUs (Figure 13). Besides the Proteobacteria, other phyla that were represented by at least $\geq 1\%$ of total OTUs, included Bacteroidetes (5.84%, 101 OTUs), Actinobacteria (4.86%, 84 OTUs), Acidobacteria (4.40%, 76 OTUs), Firmicutes (3.82%, 66 OTUs), Elusimicrobia (3.07%, 53 OTUs), Nitrospirae (2.54%, 44 OTUs), Gemmatimonadetes (1.97%, 34 OTUs), GN02 (1.16 %, 20 OTUs) and TM7 (1.16%, 20 OTUs). 50 OTUs remained unclassified at the phylum level, representing 2.89%.

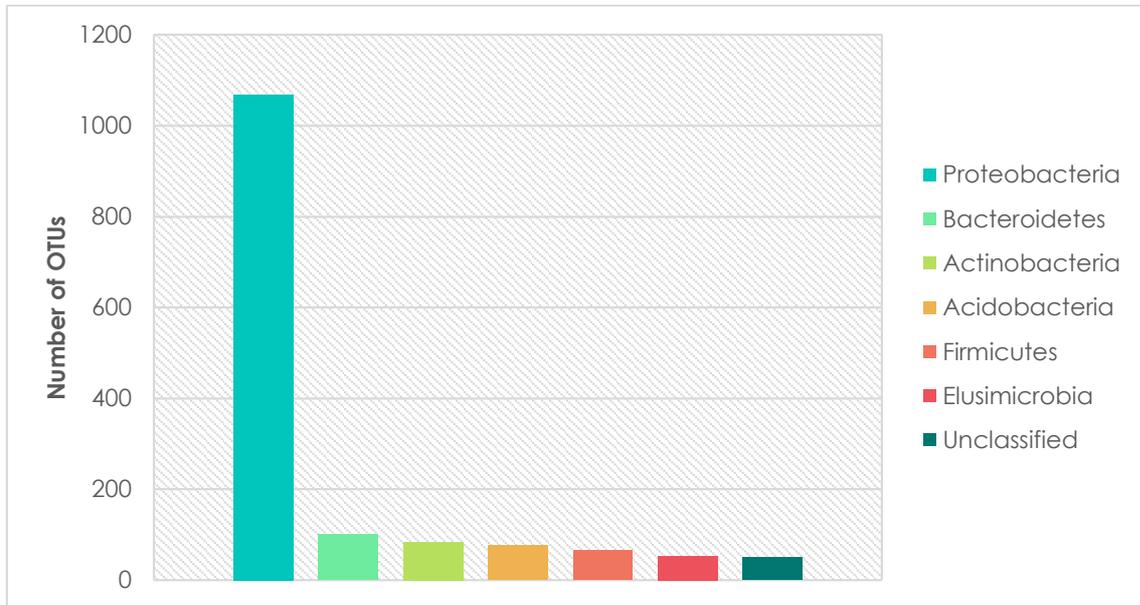


Figure 13 – Distribution of the major phyla (with more than 3%) and unclassified OTUs at phylum level. The 6 groups attending, represent 83,81% of all the phyla in the samples collected.

At Class level, a total of 79 classes were assigned. Only 13 classes were assigned at $\geq 1\%$ of total OTUs. The four dominant classes (Figure 14) were: Betaproteobacteria (16.14%, 271 OTUs), Deltaproteobacteria (15.78%, 265 OTUs), Alphaproteobacteria (15.19%, 255 OTUs) and Gammaproteobacteria (14.65%, 246 OTUs). Also, there were 8 classes with less than 3%, the Nitrospira (2.62%, 44 OTUs), Elusimicrobia (2.32%, 39 OTUs), Clostridia (2.03%, 34 OTUs), Acidimicrobia (1.97%, 33 OTUs), Bacilli (1.85%, 31 OTUs), Sphingobacteriia (1.85%, 31 OTUs), Thermoleophilia (1.85%, 31 OTUs), Acidobacteria-6 (1.61%, 27 OTUs), Saprospirae (1.25%, 21 OTUs), Actinobacteria (1.07%, 18 OTUs), Flavobacteriia (1.01%, 17 OTUs) and 3.75% remain unclassified at class level (63 OTUs).

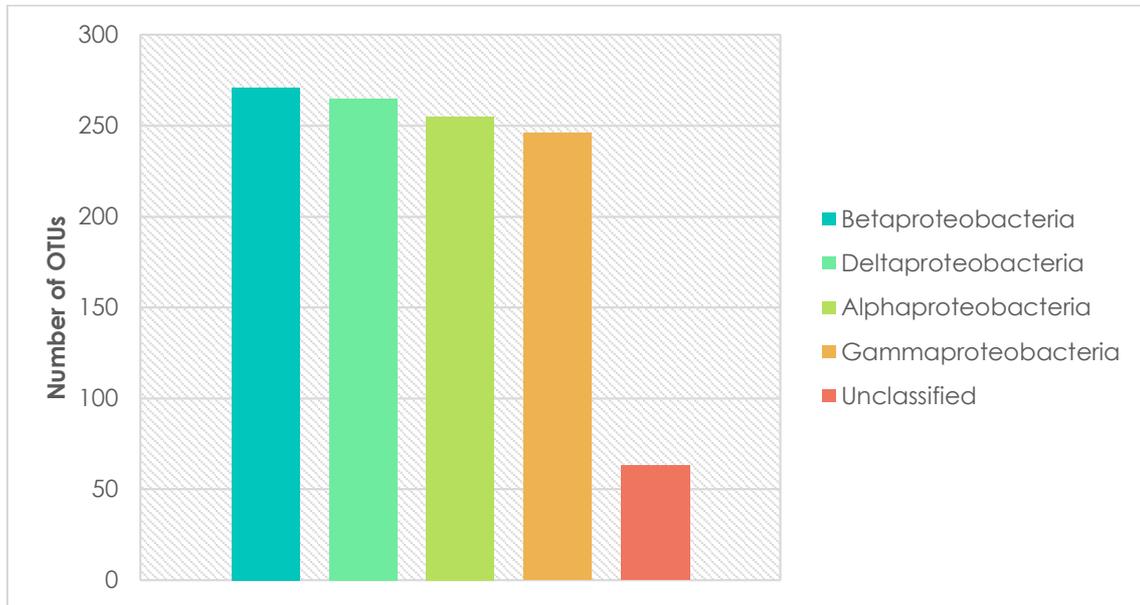


Figure 14 - Distribution of the major Classes (with more than 3%) unclassified. The 4 groups attending, represent 61,76% of all the classes in the samples collected

At order level, 117 Orders were assigned. At this level, the percentage of unclassified bacteria was 16.40% (265 OTUs). Above 3% of total OTUs, 5 orders were assigned (Figure 15), the Burkholderiales (7.43%, 120 OTUs), Myxococcales (6.56%, 106 OTUs), Xanthomonadales (3.37%, 61 OTUs), Legionellales (3.47%, 56 OTUs), Rhodospirillales (3.16%, 51 OTUs). With less than 3% of representation, the Rhizobiales (2.78%, 45 OTUs), Nitrospirales (2.72%, 44 OTUs), Rhodocyclales (2.41%, 39 OTUs), Clostridiales, Pseudomonadales and Rickettsiales (2.10%, 34 OTUs), Acidimicrobiales (2.04%, 33 OTUs). With less than 2% the Sphingobacteriales (1.92%, 31 OTUs), Spirobacillales (1.79%, 29 OTUs), Sphingomonadales (1.67%, 27 OTUs), Elusimicrobiales (1.61%, 26 OTUs), MND1 (1.49%, 24 OTUs), Syntrophobacterales (1.42%, 23 OTUs), BD7-3 (1.36%, 22 OTUs), [Saprospirales], Bacillales and Gaiellales (1.30%, 21 OTUs), Bdellovibrionales and Alteromonadales (1.24%, 20 OTUs), Actinomycetales (1.11%, 18 OTUs), Flavobacteriales, iii1-15 and Rhodobacterales (1.05%, 17 OTUs).

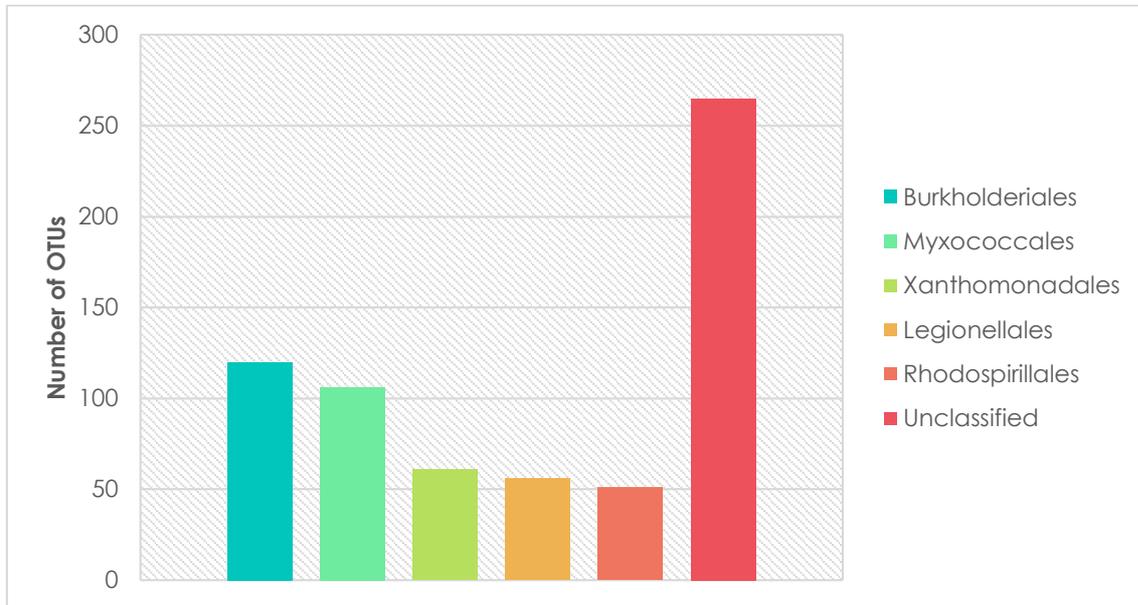


Figure 15 - Distribution of the major Orders (with more than 3%) and unclassified OTUs at Order level. The 5 groups attending, represent 24,38% of all the Orders in the samples collected.

At Family level, a total of 117 families were assigned, with 36.42% (492 OTUs) as unclassified. At $\geq 1\%$ of total OTUs, 16 families were allocated. Comamonadaceae (7.33%, 99 OTUs), Rhodospirillaceae (3.48%, 47 OTUs), Rhodocyclaceae (2.89%, 39 OTUs), Xanthomonadaceae (2.37%, 32 OTUs) and Sinobacteraceae (2.07%, 28 OTUs) were the most dominant (Figure 16) With less than 2%, Moraxellaceae (1.78%, 24 OTUs), Hyphomicrobiaceae (1.63%, 22 OTUs), Coxiellaceae and Syntrophobacteraceae (1.55%, 21 OTUs), Sphingomonadaceae (1.41%, 19 OTUs), Gaiellaceae (1.26%, 17 OTUs), Legionellaceae (1.18%, 16 OTUs), Clostridiaceae (1.18%, 16 OTUs), Haliangiaceae, Nitrospiraceae and Oxalobacteraceae (1.04%, 14 OTUs) were recorded.

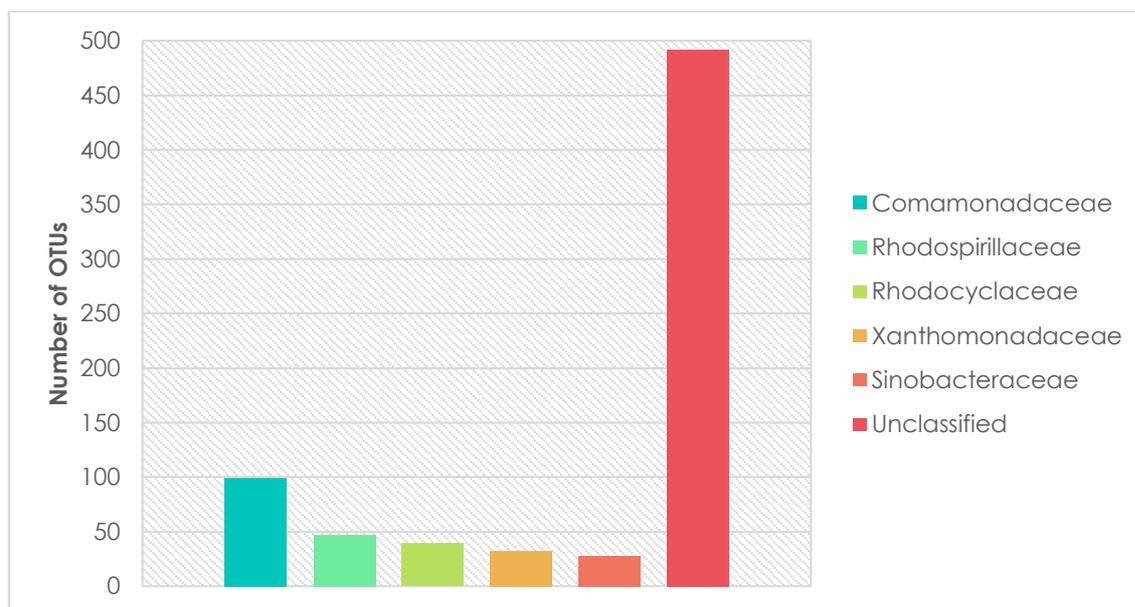


Figure 16 - Distribution of the major Families (with more than 2%) and unclassified OTUs at Family level. The 5 groups attending, represent 18,14% of all the Families in the samples collected.

In addition to these results, OTUs were assigned to Genus and Species, with the level of unclassified bacterial sequences too high for both, with 58.21 % (500 OTUs) at Genus level and 89.14% (320 OTUs) at Species level. Still, the analyses using QIIME revealed 151 OTUs assigned for Genera and 28 OTUs for Species.

In resume, the analysis of 16S rRNA libraries, based on sequence similarities, revealed that most of the bacterial communities from the samples set were affiliated with Proteobacteria phylum (62%) followed by Bacteroidetes, Actinobacteria, Acidobacteria, Firmicutes and Elusimicrobia. Proteobacteria, in the most varied environments (Kerstens et al. 2006), regarding groundwater, they are ubiquitous, not only found has the dominant phylum in groundwater (Hemme et al. 2015; Miseta et al. 2012) but also in wastewater (Liu et al. 2016) and acidic mine water (Kamika, Azizi, and Tekere 2016) samples. Proteobacteria dominance can also be explained by the strong possibility of this group playing a major key in the role biogeochemical cycle of caves (Zhou et al. 2007). Betaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Gammaproteobacteria were the major classes recorded and, at the Family level, Comamonadaceae, Rhodospirillaceae, Rhodocyclaceae, Xanthomonadaceae and Sinobacteraceae were the most abundant.

A very similar distribution was recorded in walls of karst lands close to Portugal in Altamira, Tito Bustillo, Llonin and La Garma karst caves in Spain (Schabereiter-Gurtner et al. 2002b; Schabereiter-Gurtner et al. 2004; Schabereiter-Gurtner et al. 2002a), where Proteobacteria was always the major phylum, followed by Acidobacteria. But interestingly, this phylogenetic diversity is not only present just in karst lands close to Portugal but also from distant locations such as Lechuguilla and Spider Caves in the USA (Northup et al. 2003) and Nullarbor Caves in Western Australian (Holmes et al. 2001) suggesting this spread bacterial communities for these specific environments.

OTU diversity and sample incidence

Relative abundance for Phylum, Order, Class and Family with 1% of representative - see Figures 17 to 20. The organisms closely related to the 12 more abundant OTUs and only corresponding percentage similarity of $\geq 97\%$ for the 16S rDNA bacterial partial sequences were identified manually with BLASTN (Z. Zhang et al. 2000) (Table 4).

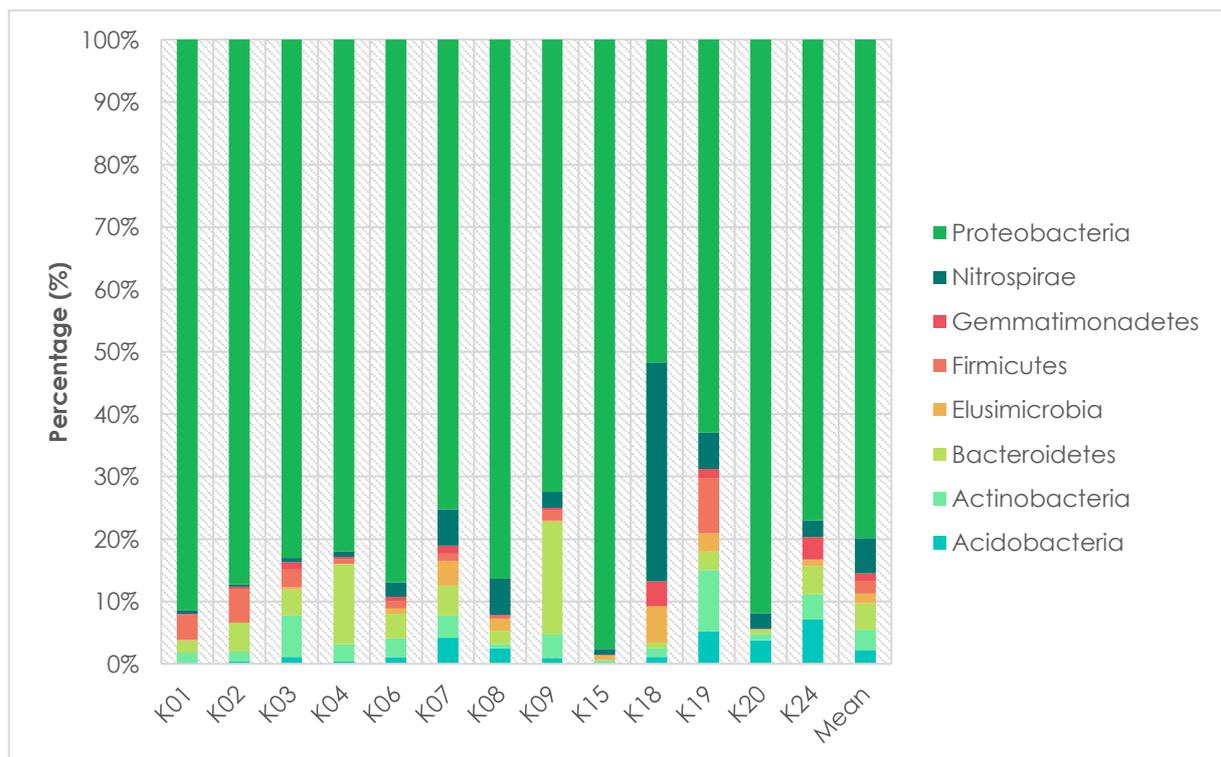


Figure 17 – Relative abundance of Phylum (with $\geq 1\%$ representation per sample).

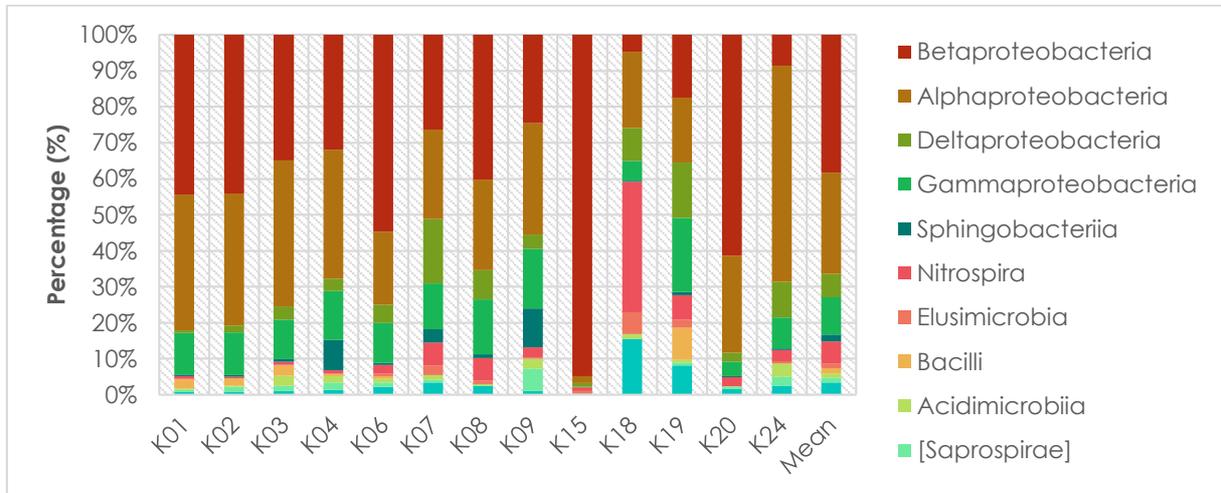


Figure 18 - Relative abundance of Class (with $\geq 1\%$ representation per sample).

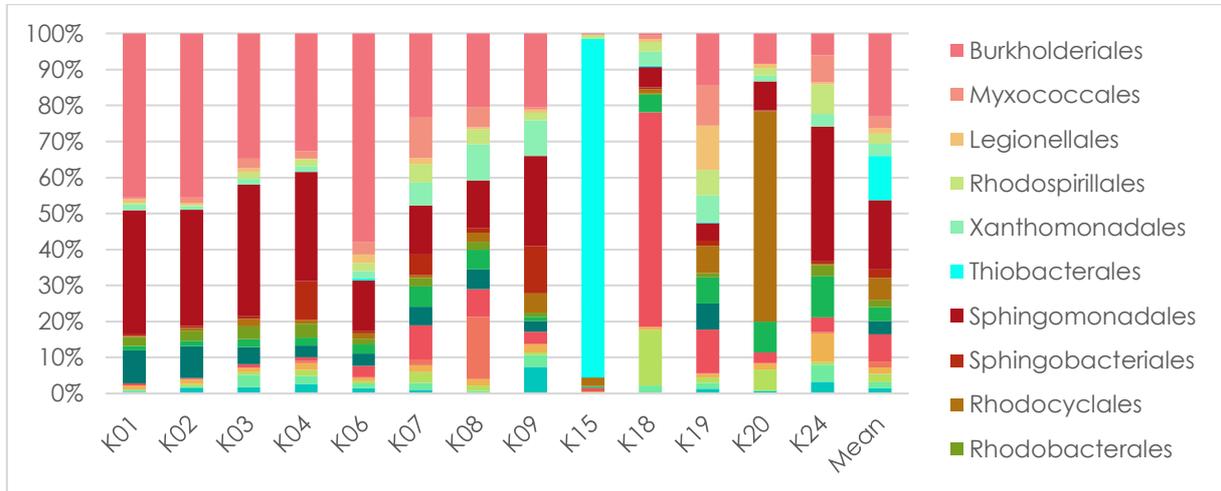


Figure 19 - Relative abundance of Order (with $\geq 1\%$ representation per sample).

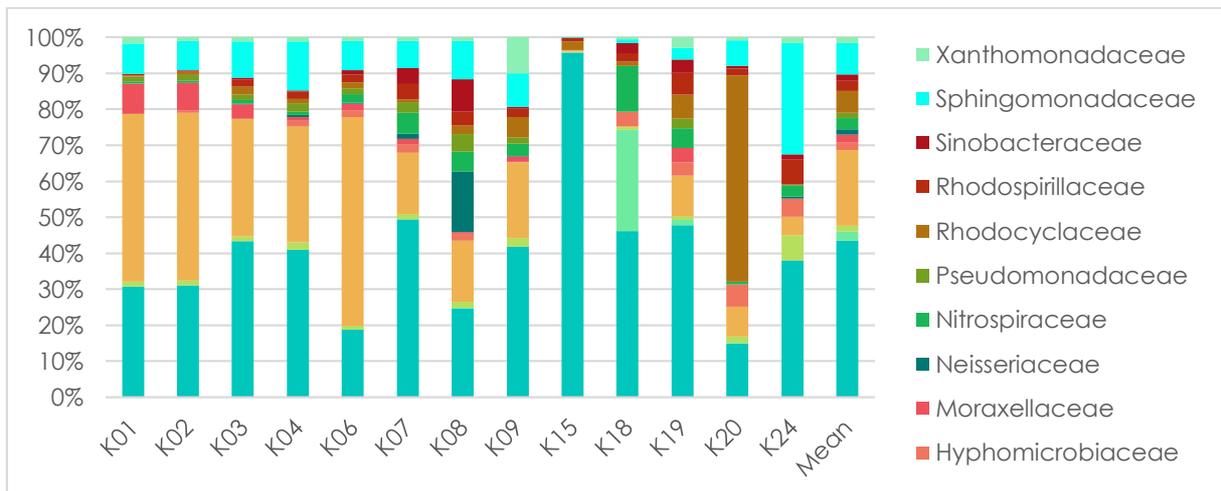


Figure 20 - Relative abundance of Family (with $\geq 1\%$ representation per sample).

Table 4 - OTU, organism, corresponding percentage similarity for the 16S rDNA bacterial partial sequences, NCBI taxonomy ID, NCBI reference sequence, Isolation source of the sample related, country/place, Pubmed ID of the paper and phylogenetic association.

OTU	ORGANISM	IDENT	NCBI TAXONOMY ID	NCBI REFERENCE SEQUENCE	ISOLATION SOURCE	COUNTRY/PLACE	PAPER (PUBMED)	PHYLOGENETIC ASSOCIATION
4	<i>Sphingopyxis rigui</i> strain 01SU5-P	99%	1282858	NR_117995.1	freshwater	Woopo wetland, South Korea	22798653	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingorhabdus.
2	<i>Limnohabitans parvus</i> strain II-B4	97%	1293052	NR_125542.1	freshwater from reservoir	Ceske Budejovice, Rimov Reservoir, Czech Republic	20061501	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
5	<i>Novosphingobium aquiterrae</i> strain E-II-3	99%	624388	NR_134104.1	underground water	Daejeon, Korea.	24994774	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium.
7	<i>Sulfuritalea hydrogenivorans</i> strain sk43H	97%	1223802	NR_113147.1	freshwater lake, stratified artificial lake	Lake Mizugaki, Japan	20709913	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfuritalea
10	<i>Limnohabitans curvus</i> strain MWH-C5	99%	323423	NR_125491.1	freshwater lake, pelagic zone	Lake Mondsee, Austria	19671731	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
55	<i>Limnohabitans planktonicus</i> strain II-D5	99%	1293045	NR_125541.1	freshwater from reservoir	Ceske Budejovice, Rimov Reservoir, Czech Republic	20061501	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
91	<i>Sphingomonas aquatilis</i> strain NBRC 16722	100%	1219039	NR_113867.1		Japan	11491350	
1721	<i>Novosphingobium aquiterrae</i> strain E-II-3	99%	48937	NR_040826.1	underground water	Daejeon, Korea	24994774	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium.
1541	<i>Sulfurisoma sedimicola</i> strain BSN1	97%	1381557	NR_125471.1	soil		24480906	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfurisoma

17	<i>Vogesella fluminis</i> strain Npb-07	99%	1069161	NR_109463.1	freshwater river	Taiwan, China	23396722	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Chromobacteriaceae; Vogesella
2109	<i>Sulfuritalea</i> <i>hydrogenivorans</i> strain sk43H	99%	1223802	NR_113147.1	freshwater lake	Lake Mizugaki, Japan	20709913	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfuritalea
29	<i>Arenimonas</i> <i>maotaiensis</i> strain YT8	99%	1446479	NR_133967.1	freshwater	Maotai section of Chishui River, China	25212225	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Arenimonas.

The most abundant OTUs were OTU-1, OTU-4 and OTU-2 with 1399, 1031 and 813 total sequences respectively. OTU-1 was assigned to order Thiobacterales (Proteobacteria, Betaproteobacteria), OTU-2 to genus *Limnohabitans* (Proteobacteria, Betaproteobacteria) and OTU-4 to order Sphingomonadales (Proteobacteria, Alphaproteobacteria).

The BLASTN (Table 4) also revealed a high similarity with *Limnohabitans planktonicus* strain II-D5 for OTU-2 and *Sphingopyxis rigui* strain 01SU5-P for the OTU-4. Moreover, the analyses of OTU-2 and OTU-4 revealed a huge dominance and co-occurrence (Annex Table 3) in samples K01, K02, K03, K06, K09 and K24, and a small occurrence at K07. Members of the genus *Limnohabitans* have been recorded in freshwaters (Newton et al. 2011), including impacted superficial waters (Lopes et al. 2016).

Strains of *Sphingopyxis* are also found in superficial freshwaters (Newton et al. 2011). This may indicate a direct influence of superficial waters in samples where these OTUs 2 and 4 are dominant which is consistent with the sampling data (most from surface waters) and unpublished data from faecal contamination at all samples, except for K24 (which is from a deep borehole distantly located, but that may have some connection with the previous samples through the many conduits, caves, etc. of the massif (Rodrigues and Fonseca 2010; Hoffmann et al. 2013).

OTU-1 was found exclusively at sample K15 indicating exclusive conditions for its occurrence. Strains of the Thiobacterales family have been linked to wastewater (Liu et al. 2016) which may also indicate a potential impact from human activity at K15. In fact, this sampling site is located in an industrial and urban area. OTU-5 was found abundantly in sample K24 and a small occurrence in the K3, K4 and K6, its sequence was similar to *Novosphingobium aquiterrae* strain E-II-3, a species isolated from a groundwater system (Lee, Kim, and Whang 2014).

OTU-7 and OTU-2109 found exclusively at K20 samples, were related to *Sulfuritalea hydrogenivorans* strain sk43H. This species has been linked to freshwater denitrification (Zeng et al. 2016) and sulfur-oxidation (Watanabe, Kojima, and Fukui 2014; Miseta et al. 2012). This may indicate a pressure from agriculture and/or industrial pollution. In fact, K20 is located in an agriculture land but this sample did not show high levels of nitrate (unpublished data). OTU-10 was mostly found in samples K03 and K06 showed the highest homology with

Limnohabitans curvus strain MWH-C5. OTU-19 also showed the highest sequence similarity with a *Limnohabitans* bacterium and this was predominantly in sample K06. In samples K03 and K06 the probability of contamination from pollution is high, as these sampling sites were at the surface level. As discussed before, this genus *Limnohabitans* has been recorded in several freshwaters (Newton et al. 2011) namely impacted (Lopes et al. 2016) and saline ones (Ko et al. 2016) which reinforce the risk for pollution impact.

OTU-11 and OTU-30 were affiliated with sequences belonging to the Nitrospirales order and both occurred at K18. Members of Nitrospirales have been recorded in groundwater anaerobic environments (Starke et al. 2017) and water contaminated with benzene and ammonium, making use of their ammonium-oxidizing capability (Wei et al. 2015). OTU-12, found in the sample K04 and K09, was related with sequences from the Sphingobacteriales order which is found in groundwater systems (Smith et al. 2015) and related to denitrifying processes (Calderer et al. 2014). Once again, these samples showed dominant phylotypes related to potential impact from surface pollution.

OTU-17, found exclusively in sample K08, was related to *Vogesella fluminis* strain Npb-07 isolated from a freshwater river (Sheu et al. 2013). OTU-29 was related with *Arenimonas maotaiensis* strain YT8 isolated from a freshwater river (Yuan et al. 2014). The OTU-91 was related to *Sphingomonas aquatilis* strain NBRC 16722 found exclusively in the sample K08F and K24F. Strains related to this species have been recorded in mineral waters (Takeuchi et al. 2001) but also in wastewaters (Huo et al. 2011). OTU-1541, related with *Sulfurisoma sediminicola* strain BSN1 K20, isolated from a freshwater lake (Kojima and Fukui 2014).

Relating data from sequencing and Cluster analysis (Figure 10 and Figure 11) and data from taxonomic affiliation it was possible to track characteristic dominant phylotypes from these samples (that are near or at the surface) from cluster A and they were associated with organisms belonging to the genera *Limnohabitans*, *Sphingopyxis* and members of the order Sphingobacteriales related to superficial waters and also waters impacted from pollution. Definitely, it is possible to make this connection between these samples and a higher risk for pollution intrusion. As discussed above, samples K01, K02, K03, K04, K06 and K09 have shown faecal contamination (unpublished data from Karstrisk project) suggesting an impact from exogenous superficial effluents. In fact, microbial contamination is frequent in karst springs

worldwide (Pronk, Goldscheider, and Zopfi 2006; Dussart-Baptista et al. 2007; Moss, Nocker, and Snyder 2011) and members of the dominant OTUs were already found in pollution impacted water bodies (Jordaan and Bezuidenhout 2016).

A hypothesis supported by other studies, even though not directly related with the groundwater bacterial communities, showed a direct impact on land use/land cover and the bacterial communities present (Jangid et al. 2008; Lear and Lewis 2009). In 2008, Jangid and colleagues demonstrated a direct impact of land use/land cover (long-term agricultural and forest areas) in soil bacterial communities, that were significantly altered in agricultural areas (Jangid et al. 2008). Lear and Lewis also demonstrated that whole bacterial community were sensitive descriptors of ecological conditions and capable of differentiating between sites exposed to different land uses (Lear and Lewis 2009).

In the case of the sample K18 and K19, both collected close to an industrial factory of minerals and lime producer, showed the highest number of OTU richness, an appearance of Nitrospirales and Nitrospira-like bacteria have been observed in the dominant populations of the nitrifying-activated sludge of industrial wastewater treatment plants (sewage with high ammonia concentration), to transform ammonia to nitrite (S. Zhang, Yang, and Singh 2014).

4. Conclusion

In Portugal, there is a lack of previous studies concerning the phylogenetic composition and diversity of bacterial communities in groundwater systems, namely from the Estremenho karst massif. This study gathered new information from results obtained using two culture-independent molecular approaches (PCR-DGGE and pyrosequencing). DGGE showed to be a valuable screening method and more affordable, providing a reliable idea on the overall diversity of samples. However, the next-generation approach (pyrosequencing) provided much more information that is incomparably more representative for the research purpose of studying the composition and diversity of bacterial communities in these environments, namely at the phylogenetic level.

As in many other studies on groundwater worldwide, in the present work Proteobacteria revealed to be the most abundant phylum, dominating the obtained OTUs. It was distributed by the orders Thiobacterales (Betaproteobacteria), Rhodocyclales (Betaproteobacteria), Burkholderiales (Betaproteobacteria), Sphingomonadales (Alphaproteobacteria), Xanthomonadales (Gammaproteobacteria), Neisseriales (Betaproteobacteria) and Acidiferrobacterales (Gammaproteobacteria). Other less abundant phyla included the Bacteroidetes (Sphingobacteria), Actinobacteria (Acidimicrobiia), Acidobacteria, Firmicutes (Bacilli), Elusimicrobia and Nitrospirae (Nitrospira).

In addition to the composition and diversity revealed in this study, data suggested an intimate connection between surface and groundwater in many samples, not only because of the geologic characteristics of the terrain, highly susceptible to anthropogenic contamination from surface points and diffuse sources of pollution but also due to the land use/land cover. Terrain characterised by intensive agriculture, livestock and industries (textiles, leather plants, olive oil processing plants) in some of the sampling sites, suggested a relationship with the composition of bacterial communities of the groundwater. Samples collected at the surface or near the surface indicated a strong influence of anthropogenic pollution, also corroborated by the dominant *taxa* affiliated with genera *Limnohabitans* and *Sphingopyxis* and members of the order Sphingobacteriales, commonly related to superficial and polluted waters. These results indicate a relationship between samples and a higher risk for pollution intrusion from

the land use. This highlights the importance of dominant *taxa* in bacterial communities to be used as water quality bioindicators.

The study also showed that the sampled springs, wells and boreholes can provide an important first screening about the diversity and function of microbial assemblages in groundwater from this karst massif. Considering the inexistence of information of bacterial diversity in the karst areas from Portugal, this study, in spite of based on data from a one-time sampling campaign, represents a critical knowledge gap, for a better understanding of these essential habitats. Therefore, it may be used as an important baseline reference for further studies, that should include time-series data, at least seasonally, due to variations in groundwater levels.

5. References

- Abbazadegan, M, M S Huber, C P Gerba, and I L Pepper. 1993. "Detection of Enteroviruses in Groundwater Using the Polymerase Chain Reaction." *Applied and Environmental Microbiology* 59 (5): 1318–24. doi:10.1016/0732-8893(88)90014-4.
- Almeida, C.A., J.L. Mendonça, M.R. Jesus, and A.J. Gomes. 2000. "Sistemas Aquíferos de Portugal Continental: Lisboa." *Centro de Geologia Da Universidade de Lisboa/ Instituto Da Água*.
- Anantharaman, Karthik, Christopher T. Brown, Laura A. Hug, Itai Sharon, Cindy J. Castelle, Alexander J. Probst, Brian C. Thomas, et al. 2016. "Thousands of Microbial Genomes Shed Light on Interconnected Biogeochemical Processes in an Aquifer System." *Nature Communications* 7 (October): 13219. doi:10.1038/ncomms13219.
- Angly, Florent, Beltran Rodriguez-Brito, David Bangor, Pat McNairnie, Mya Breitbart, Peter Salamon, Ben Felts, James Nulton, Joseph Mahaffy, and Forest Rohwer. 2005. "PHACCS, an Online Tool for Estimating the Structure and Diversity of Uncultured Viral Communities Using Metagenomic Information." *BMC Bioinformatics* 6 (1): 41. doi:10.1186/1471-2105-6-41.
- Ansorge, WJ. 2015. "Next Generation DNA Sequencing (II): Techniques, Applications." *Journal of Next Generation Sequencing & Applications* 1 (S1). doi:10.4172/2469-9853.S1-005.
- APA. 2015a. "Plano de Gestão de Região Hidrográfica - Região Hidrográfica Do Tejo E Ribeiras Do Oeste (RH5)," 55.
- APA. 2015b. "Plano Nacional Da Água," 1–259.
- APA. 2015c. "Relatório Do Estado Do Ambiente." Agência Portuguesa do Ambiente.
- Axenov-Gibanov, Denis V., Irina V. Voytsekhovskaya, Bogdan T. Tokovenko, Eugeny S. Protasov, Stanislav V. Gamaiunov, Yuriy V. Rebets, Andriy N. Luzhetskyy, and Maxim A. Timofeyev. 2016. "Actinobacteria Isolated from an Underground Lake and Moonmilk Speleothem from the Biggest Conglomeratic Karstic Cave in Siberia as Sources of Novel Biologically Active Compounds." Edited by Krishnendu Acharya. *PLOS ONE* 11 (2): e0149216. doi:10.1371/journal.pone.0149216.
- Biemans, H., I. Haddeland, P. Kabat, F. Ludwig, R. W. A. Hutjes, J. Heinke, W. von Bloh, and D. Gerten. 2011. "Impact of Reservoirs on River Discharge and Irrigation Water Supply during the 20th Century." *Water Resources Research* 47 (3): n/a-n/a. doi:10.1029/2009WR008929.
- Braun, Burga, Josephin Schröder, Henrik Knecht, and Ulrich Szewzyk. 2016. "Unraveling the Microbial Community of a Cold Groundwater Catchment System." *Water Research* 107 (December): 113–26. doi:10.1016/j.watres.2016.10.040.
- Calderer, M., V. Martí, J. de Pablo, M. Guivernau, F.X. Prenafeta-Boldú, and M. Viñas. 2014. "Effects of

- Enhanced Denitrification on Hydrodynamics and Microbial Community Structure in a Soil Column System." *Chemosphere* 111 (September): 112–19. doi:10.1016/j.chemosphere.2014.03.033.
- Caldwell, E. L. 1937. "Pollution Flow from Pit Latrines When an Impervious Stratum Closely Underlies the Flow." *Journal of Infectious Diseases* 61 (3): 270–88. doi:10.1093/infdis/61.3.270.
- Caporaso, J Gregory, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D Bushman, Elizabeth K Costello, Noah Fierer, et al. 2010. "QIIME Allows Analysis of High-Throughput Community Sequencing Data." *Nature Methods* 7 (5): 335–36. doi:10.1038/nmeth.f.303.
- Cleary, Daniel F. R., Kornelia Smalla, Leda C. S. Mendonça-Hagler, and Newton C. M. Gomes. 2012. "Assessment of Variation in Bacterial Composition among Microhabitats in a Mangrove Environment Using DGGE Fingerprints and Barcoded Pyrosequencing." Edited by Jacques Ravel. *PLoS ONE* 7 (1): e29380. doi:10.1371/journal.pone.0029380.
- Cleary, Daniel F R, Nicole J. de Voogd, Ana R M Polonia, Rossana Freitas, and Newton C M Gomes. 2015. "Composition and Predictive Functional Analysis of Bacterial Communities in Seawater, Sediment and Sponges in the Spermonde Archipelago, Indonesia." *Microbial Ecology* 70 (4): 889–903. doi:10.1007/s00248-015-0632-5.
- Danielopol, Dan A N L, Christian Griebler, Amara Gunatilaka, and Jos Notenboom. 2003. "Present State and Future Prospects for Groundwater Ecosystems." *Environmental Conservation* 30 (2). Cambridge University Press: 104–30. doi:10.1017/S0376892903000.
- Drew, David Philip, and Heinz Hötzl. 1999. *Karst Hydrogeology and Human Activities: Impacts, Consequences and Implications*. Book. Iah International Contributions to Hydrogeology Series. A.A. Balkema.
- Dussart-Baptista, L., J. Bodilis, S. Barray, N. Frébourg, M. Fournier, J. P. Dupont, and T. Jouenne. 2007. "Recurrent Recovery of Pseudomonas Oryzihabitans Strains in a Karstified Chalk Aquifer." *Water Research* 41 (1): 111–17. doi:10.1016/j.watres.2006.10.007.
- Ehn, Maria, Nader Nourizad, Kristina Bergström, Afshin Ahmadian, Pål Nyrén, Joakim Lundeberg, and Sophia Hober. 2004. "Toward Pyrosequencing on Surface-Attached Genetic Material by Use of DNA-Binding Luciferase Fusion Proteins." *Analytical Biochemistry* 329 (1): 11–20. doi:10.1016/j.ab.2004.02.005.
- Ellegaard, Kirsten M., and Philipp Engel. 2016. "Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota." *Frontiers in Microbiology* 7 (September). doi:10.3389/fmicb.2016.01475.
- Ford, D, and P D Williams. 2007. *Karst Hydrogeology and Geomorphology*. Book. Edited by Chapman and Hall. Wiley.

- Gleick, Peter H. 1993. *Water in Crisis: A Guide to the World's Fresh Water Resources*. Oxford University Press. <https://books.google.com/books?id=NigDkxQOKSAC&pgis=1>.
- Goldscheider, Nico, Daniel Hunkeler, and Pierre Rossi. 2006. "Review: Microbial Biocenoses in Pristine Aquifers and an Assessment of Investigative Methods." *Hydrogeology Journal* 14 (6): 926–41. doi:10.1007/s10040-005-0009-9.
- Hammer, Ø., David A. T. Harper, P. D. Ryan, and Paul D. Ryan. 2001. "PAST: PALEONTOLOGICAL STATISTICS SOFTWARE PACKAGE FOR EDUCATION AND DATA ANALYSIS." *Palaeontologia Electronica* 4 (1): 9. doi:10.1016/j.bcp.2008.05.025.
- Handelsman, Jo. 2004. "Metagenomics: Application of Genomics to Uncultured Microorganisms." *Microbiology and Molecular Biology Reviews: MMBR* 68 (4): 669–85. doi:10.1128/MMBR.68.4.669-685.2004.
- Hemme, Christopher L., Qichao Tu, Zhou Shi, Yujia Qin, Weimin Gao, Ye Deng, Joy D. Van Nostrand, et al. 2015. "Comparative Metagenomics Reveals Impact of Contaminants on Groundwater Microbiomes." *Frontiers in Microbiology* 6 (October). doi:10.3389/fmicb.2015.01205.
- Hoffmann, Dirk L., Alistair W G Pike, Karine Wainer, and João Zilhão. 2013. "New U-Series Results for the Speleogenesis and the Palaeolithic Archaeology of the Almonda Karstic System (Torres Novas, Portugal)." *Quaternary International* 294. Elsevier Ltd and INQUA: 168–82. doi:10.1016/j.quaint.2012.05.027.
- Holmes, A J, N A Tujula, M Holley, A Contos, J M James, P Rogers, and M R Gillings. 2001. "Phylogenetic Structure of Unusual Aquatic Microbial Formations in Nullarbor Caves, Australia." *Environmental Microbiology* 3 (4): 256–64. doi:11359511.
- Hugenholtz, P, B M Goebel, and N R Pace. 1998. "Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity." *Journal of Bacteriology*. doi:0021-9193/98/\$04.00+0.
- Huo, Y.-Y., X.-W. Xu, S.-P. Liu, H.-L. Cui, X. Li, and M. Wu. 2011. "Sphingomonas Rubra Sp. Nov., Isolated from Bioreactor Wastewater." *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 61 (5): 1028–32. doi:10.1099/ijs.0.020958-0.
- ICNF. 2015. "Parque Natural Das Serras de Aire E Candeeiros." <http://www.icnf.pt/portal/turnatur/visit-ap/pn/pnsac/inf-ger>.
- INAG. 2005. "Relatório Síntese Sobre a Caracterização Das Regiões Hidrográficas Prevista Na Directiva-Quadro Da Água."
- Jackson, Colin R, Colin R Jackson, Eric E Roden, Eric E Roden, Perry F Churchill, and Perry F Churchill. 2000. "Denaturing Gradient Gel Electrophoresis Can Fail to Separate 16S rDNA Fragments with

- Multiple Base Differences.” *Molecular Biology Today* 1 (2): 49–51.
- Jangid, Kamlesh, Mark A. Williams, Alan J. Franzluebbers, Jamie S. Sanderlin, Jaxk H. Reeves, Michael B. Jenkins, Dinku M. Endale, David C. Coleman, and William B. Whitman. 2008. “Relative Impacts of Land-Use, Management Intensity and Fertilization upon Soil Microbial Community Structure in Agricultural Systems.” *Soil Biology and Biochemistry* 40 (11): 2843–53. doi:10.1016/j.soilbio.2008.07.030.
- Jordaan, K., and C. C. Bezuidenhout. 2016. “Bacterial Community Composition of an Urban River in the North West Province, South Africa, in Relation to Physico-Chemical Water Quality.” *Environmental Science and Pollution Research* 23 (6): 5868–80. doi:10.1007/s11356-015-5786-7.
- Kamika, I., S. Azizi, and M. Tekere. 2016. “Microbial Profiling of South African Acid Mine Water Samples Using next Generation Sequencing Platform.” *Applied Microbiology and Biotechnology* 100 (13): 6069–79. doi:10.1007/s00253-016-7428-5.
- Kerstens, Karel, Paul De Vos, Monique Gillis, Jean Swings, Peter Vandamme, and Erko Stackebrandt. 2006. “Introduction to the Proteobacteria.” In *The Prokaryotes*, 3–37. New York, NY: Springer New York. doi:10.1007/0-387-30745-1_1.
- Keswick, Bruce H., De-Shin Wang, and Charles P. Gerba. 1982. “The Use of Microorganisms as Ground-Water Tracers: A Review.” *Ground Water* 20 (2): 142–49. doi:10.1111/j.1745-6584.1982.tb02741.x.
- Ko, Myoung-Soo, Kyungjin Cho, Dawoon Jeong, and Seunghak Lee. 2016. “Identification of the Microbes Mediating Fe Reduction in a Deep Saline Aquifer and Their Influence during Managed Aquifer Recharge.” *Science of The Total Environment* 545–546 (March): 486–92. doi:10.1016/j.scitotenv.2015.12.106.
- Koeppel, A. F., and M. Wu. 2013. “Surprisingly Extensive Mixed Phylogenetic and Ecological Signals among Bacterial Operational Taxonomic Units.” *Nucleic Acids Research* 41 (10): 5175–88. doi:10.1093/nar/gkt241.
- Kojima, H., and M. Fukui. 2014. “Sulfurisoma Sediminicola Gen. Nov., Sp. Nov., a Facultative Autotroph Isolated from a Freshwater Lake.” *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 64 (Pt 5): 1587–92. doi:10.1099/ijls.0.057281-0.
- Kuczynski, Justin, Jesse Stombaugh, William Anton Walters, Antonio González, J. Gregory Caporaso, and Rob Knight. 2011. “Using QIIME to Analyze 16S rRNA Gene Sequences from Microbial Communities.” In *Current Protocols in Bioinformatics*. Hoboken, NJ, USA: John Wiley & Sons, Inc. doi:10.1002/0471250953.bi1007s36.
- LANDYDYN. 2014. *USO E OCUPAÇÃO DO SOLO EM PORTUGAL CONTINENTAL: Avaliação E Cenários*

- Futuros*. Edited by Direção-Geral do Território (DGT). Lisboa.
- Lear, G., and G.D. Lewis. 2009. "Impact of Catchment Land Use on Bacterial Communities within Stream Biofilms." *Ecological Indicators* 9 (5): 848–55. doi:10.1016/j.ecolind.2008.10.001.
- Lee, J.-C., S.-G. Kim, and K.-S. Whang. 2014. "Novosphingobium Aquiterrae Sp. Nov., Isolated from Ground Water." *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 64 (Pt 9): 3282–87. doi:10.1099/ijs.0.060749-0.
- Lemarchand, Karine, Luke Masson, and Roland Brousseau. 2004. "Molecular Biology and DNA Microarray Technology for Microbial Quality Monitoring of Water." *Critical Reviews in Microbiology* 30 (3): 145–72. doi:10.1080/10408410490435142.
- Lerner, David N., and Bob Harris. 2009. "The Relationship between Land Use and Groundwater Resources and Quality." *Land Use Policy* 26 (December): S265–73. doi:10.1016/j.landusepol.2009.09.005.
- Life Science. 2014. "<http://www.454.com/>." 2015. <http://www.454.com/>.
- Liu, Tang, Shufeng Liu, Maosheng Zheng, Qian Chen, and Jinren Ni. 2016. "Performance Assessment of Full-Scale Wastewater Treatment Plants Based on Seasonal Variability of Microbial Communities via High-Throughput Sequencing." Edited by Andrew C Singer. *PLOS ONE* 11 (4): e0152998. doi:10.1371/journal.pone.0152998.
- Lopes, Fabyano Alvares Cardoso, Elisa Caldeira Pires Catão, Renata Henrique Santana, Anderson de Souza Cabral, Rodolfo Paranhos, Thiago Pessanha Rangel, Carlos Eduardo de Rezende, et al. 2016. "Microbial Community Profile and Water Quality in a Protected Area of the Caatinga Biome." Edited by John J. Kelly. *PLOS ONE* 11 (2): e0148296. doi:10.1371/journal.pone.0148296.
- Lu, Xiaoxia, John T. Wilson, and Donald H. Kampbell. 2006. "Relationship between Geochemical Parameters and the Occurrence of Dehalococcoides DNA in Contaminated Aquifers." *Water Resources Research* 42 (8): n/a-n/a. doi:10.1029/2005WR004283.
- Margulies, Marcel, Michael Egholm, William E Altman, Said Attiya, Joel S Bader, Lisa A Bembien, Jan Berka, et al. 2005. "Genome Sequencing in Microfabricated High-Density Picolitre Reactors." *Nature* 437 (7057): 376–80. doi:10.1038/nature03959.
- Marian Ijzerman, M., Daniel R. Dahling, and G. Shay Fout. 1997. "A Method to Remove Environmental Inhibitors prior to the Detection of Waterborne Enteric Viruses by Reverse Transcription-Polymerase Chain Reaction." *Journal of Virological Methods* 63 (1–2): 145–53. doi:10.1016/S0166-0934(96)02123-4.
- Metzker, Michael L. 2007. "Sequencing Technologies — the next Generation." *Methods in Molecular Biology (Clifton, N.J.)* 373 (January): 1–14. doi:10.1385/1-59745-377-3:1.

- Meybeck, M. 2003. "Global Analysis of River Systems: From Earth System Controls to Anthropocene Syndromes." *Philosophical Transactions of the Royal Society B: Biological Sciences* 358 (1440): 1935–55. doi:10.1098/rstb.2003.1379.
- Miseta, Roland, Márton Palatinszky, Judit Makk, Károly Márialigeti, and Andrea K. Borsodi. 2012. "Phylogenetic Diversity of Bacterial Communities Associated with Sulfurous Karstic Well Waters of a Hungarian Spa." *Geomicrobiology Journal* 29 (2): 101–13. doi:10.1080/01490451.2011.558563.
- Moss, Joseph A., Andreas Nocker, and Richard A. Snyder. 2011. "Microbial Characteristics of a Submerged Karst Cave System in Northern Florida." *Geomicrobiology Journal* 28 (8): 719–31. doi:10.1080/01490451.2010.517235.
- Muyzer, Gerard. 1999. "DGGE/TGGE a Method for Identifying Genes from Natural Ecosystems." *Current Opinion in Microbiology* 2 (3): 317–22. doi:10.1016/S1369-5274(99)80055-1.
- Newton, R. J., S. E. Jones, A. Eiler, K. D. McMahon, and S. Bertilsson. 2011. "A Guide to the Natural History of Freshwater Lake Bacteria." *Microbiology and Molecular Biology Reviews* 75 (1): 14–49. doi:10.1128/MMBR.00028-10.
- Northup, Diana E, Susan M Barns, Laura E Yu, Michael N Spilde, Rachel T Schelble, Kathleen E Dano, Laura J Crossey, et al. 2003. "Diverse Microbial Communities Inhabiting Ferromanganese Deposits in Lechuguilla and Spider Caves." *Environmental Microbiology* 5 (11): 1071–86. <http://www.ncbi.nlm.nih.gov/pubmed/14641587>.
- Pielke, Roger a., Andy Pitman, Dev Niyogi, Rezaul Mahmood, Clive McAlpine, Faisal Hossain, Kees Klein Goldewijk, et al. 2011. "Land Use/land Cover Changes and Climate: Modeling Analysis and Observational Evidence." *Wiley Interdisciplinary Reviews: Climate Change* 2 (6): 828–50. doi:10.1002/wcc.144.
- Pronk, Michiel, Nico Goldscheider, and Jakob Zopfi. 2006. "Dynamics and Interaction of Organic Carbon, Turbidity and Bacteria in a Karst Aquifer System." *Hydrogeology Journal* 14 (4): 473–84. doi:10.1007/s10040-005-0454-5.
- Reboleira, Ana Sofia, Paulo Borges, Fernando Gonçalves, Artur Serrano, and Pedro Oromí. 2011. "The Subterranean Fauna of a Biodiversity Hotspot Region - Portugal: An Overview and Its Conservation." *International Journal of Speleology* 40 (1): 23–37. doi:10.5038/1827-806X.40.1.4.
- Ribeiro, Luís (Instituto Superior Técnico), and Luís Veiga (Universidade Nova de Lisboa) Cunha. 2010. "Portuguese Groundwater Report – Easac Wg on the Role of Groundwater in the Water Resources Policy of Southern Eu Member States." www.easac.eu/fileadmin/PDF_s/reports_statements/Portugal_Groundwater_country_report.p

df.

- Ritter, Len, Keith Solomon, Paul Sibley, Ken Hall, Patricia Keen, Gevan Mattu, and Beth Linton. 2002. "Sources, Pathways, and Relative Risks of Contaminants in Surface Water and Groundwater: A Perspective Prepared for the Walkerton Inquiry." *Journal of Toxicology and Environmental Health. Part A* 65 (1): 1–142. <http://www.ncbi.nlm.nih.gov/pubmed/11809004>.
- Rodrigues, Maria Luísa, and André Fonseca. 2010. "Geoheritage Assessment Based on Large-Scale Geomorphological Mapping: Contributes from a Portuguese Limestone Massif Example." *Geomorphologie: Relief, Processus, Environnement* 16 (2): 189–98. doi:10.4000/geomorphologie.7924.
- Schabereiter-Gurtner, Claudia, Cesareo Saiz-Jimenez, Guadalupe Pinar, Werner Lubitz, and Sabine Rölleke. 2004. "Phylogenetic Diversity of Bacteria Associated with Paleolithic Paintings and Surrounding Rock Walls in Two Spanish Caves (Llonch and La Garma)." *FEMS Microbiology Ecology* 47 (2): 235–47. doi:10.1016/S0168-6496(03)00280-0.
- Schabereiter-Gurtner, Claudia, Cesareo Saiz-Jimenez, Guadalupe Piñar, Werner Lubitz, and Sabine Rölleke. 2002a. "Altamira Cave Paleolithic Paintings Harbor Partly Unknown Bacterial Communities." *FEMS Microbiology Letters* 211 (1): 7–11. <http://www.ncbi.nlm.nih.gov/pubmed/12052543>.
- Schabereiter-Gurtner, Claudia, Saiz-Jimenez, Cesareo, Piñar, Guadalupe, Lubitz, Werner, Rölleke, Sabine. 2002b. "Phylogenetic 16S rRNA Analysis Reveals the Presence of Complex and Partly Unknown Bacterial Communities in Tito Bustillo Cave, Spain, and on Its Palaeolithic Paintings." *Environmental Microbiology* 4 (7): 392–400. <http://www.ncbi.nlm.nih.gov/pubmed/12123475>.
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, et al. 2009. "Introducing Mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities." *Applied and Environmental Microbiology* 75 (23): 7537–41. doi:10.1128/AEM.01541-09.
- Sheu, S.-Y., J.-C. Chen, C.-C. Young, and W.-M. Chen. 2013. "Vogesella Fluminis Sp. Nov., Isolated from a Freshwater River, and Emended Description of the Genus Vogesella." *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 63 (Pt 8): 3043–49. doi:10.1099/ijs.0.048629-0.
- Smith, Renee J., James S. Paterson, Cally A. Sibley, John L. Hutson, and James G. Mitchell. 2015. "Putative Effect of Aquifer Recharge on the Abundance and Taxonomic Composition of Endemic Microbial Communities." Edited by Jean-François Humbert. *PLOS ONE* 10 (6): e0129004. doi:10.1371/journal.pone.0129004.

- Sophocleous, Marios. 2002. "Interactions between Groundwater and Surface Water: The State of the Science." *Hydrogeology Journal* 10 (1): 52–67. doi:10.1007/s10040-001-0170-8.
- Starke, Robert, Martina Müller, Michael Gaspar, Manja Marz, Kirsten Küsel, Kai Uwe Totsche, Martin von Bergen, and Nico Jehmlich. 2017. "Candidate Brocadiales Dominates C, N and S Cycling in Anoxic Groundwater of a Pristine Limestone-Fracture Aquifer." *Journal of Proteomics* 152 (January): 153–60. doi:10.1016/j.jprot.2016.11.003.
- Stein, Heide, Claudia Kellermann, Susanne I. Schmidt, Heike Brielmann, Christian Steube, Sven E. Berkhoff, Andreas Fuchs, Hans Jürgen Hahn, Barbara Thulin, and Christian Griebler. 2010. "The Potential Use of Fauna and Bacteria as Ecological Indicators for the Assessment of Groundwater Quality." *J. Environ. Monit.* 12 (1): 242–54. doi:10.1039/B913484K.
- Stewart, Eric J. 2012. "Growing Unculturable Bacteria." *Journal of Bacteriology*. doi:10.1128/JB.00345-12.
- Sun, Yijun, Yunpeng Cai, Susan M. Huse, Rob Knight, William G. Farmerie, Xiaoyu Wang, and Volker Mai. 2012. "A Large-Scale Benchmark Study of Existing Algorithms for Taxonomy-Independent Microbial Community Analysis." *Briefings in Bioinformatics* 13 (1): 107–21. doi:10.1093/bib/bbr009.
- Takeuchi, M, J H Yoon, Y H Park, Y R Pyun, Y K Shin, and J S Lee. 2001. "Sphingomonas Aquatilis Sp. Nov., Sphingomonas Koreensis Sp. Nov., and Sphingomonas Taejonensis Sp. Nov., Yellow-Pigmented Bacteria Isolated from Natural Mineral Water." *International Journal of Systematic and Evolutionary Microbiology* 51 (4): 1491–98. doi:10.1099/00207713-51-4-1491.
- Techtmann, Stephen M., and Terry C. Hazen. 2016. "Metagenomic Applications in Environmental Monitoring and Bioremediation." *Journal of Industrial Microbiology & Biotechnology* 43 (10): 1345–54. doi:10.1007/s10295-016-1809-8.
- Thomas, Torsten, Jack Gilbert, and Folker Meyer. 2012. "Metagenomics - a Guide from Sampling to Data Analysis." *Microbial Informatics and Experimentation* 2 (1): 3. doi:10.1186/2042-5783-2-3.
- Tomlinson, Moya, Andrew J. Boulton, Peter J. Hancock, and Peter G. Cook. 2007. "Deliberate Omission or Unfortunate Oversight: Should Stygofaunal Surveys Be Included in Routine Groundwater Monitoring Programs?" *Hydrogeology Journal* 15 (7): 1317–20. doi:10.1007/s10040-007-0211-z.
- United Nations. 2003. "Water for People, Water for Life." Paris.
- Vörösmarty, C J, P B McIntyre, M O Gessner, D Dudgeon, A Prusevich, P Green, S Glidden, et al. 2010. "Global Threats to Human Water Security and River Biodiversity." *Nature* 467 (7315). Nature Publishing Group: 555–61. doi:10.1038/nature09440.
- Watanabe, Tomohiro, Hisaya Kojima, and Manabu Fukui. 2014. "Complete Genomes of Freshwater

- Sulfur Oxidizers *Sulfuricella Denitrificans* skB26 and *Sulfuritalea Hydrogenivorans* sk43H: Genetic Insights into the Sulfur Oxidation Pathway of Betaproteobacteria." *Systematic and Applied Microbiology* 37 (6): 387–95. doi:10.1016/j.syapm.2014.05.010.
- Wei, Manman, Falk Harnisch, Carsten Vogt, Jörg Ahlheim, Thomas R. Neu, and Hans H. Richnow. 2015. "Harvesting Electricity from Benzene and Ammonium-Contaminated Groundwater Using a Microbial Fuel Cell with an Aerated Cathode." *RSC Adv.* 5 (7): 5321–30. doi:10.1039/C4RA12144A.
- Williams, Paul. 2008. "World Heritage Caves and Karst." Article. *IUCN, Gland, Switzerland*, 57.
- Wilson, I.G. 1997. "Inhibition and Facilitation of Nucleic Acid Amplification." *Applied and Environmental Microbiology* 63 (10): 3741–51. <http://www.ncbi.nlm.nih.gov/pubmed/9327537>.
- Yuan, X., Y. Nogi, X. Tan, R.-G. Zhang, and J. Lv. 2014. "Arenimonas Maotaiensis Sp. Nov., Isolated from Fresh Water." *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 64 (Pt 12): 3994–4000. doi:10.1099/ijs.0.067769-0.
- Zeng, Xiangyong, Takahiro Hosono, Hiroto Ohta, Takuro Niidome, Jun Shimada, and Shigeru Morimura. 2016. "Comparison of Microbial Communities inside and outside of a Denitrification Hotspot in Confined Groundwater." *International Biodeterioration & Biodegradation* 114 (October): 104–9. doi:10.1016/j.ibiod.2016.05.019.
- Zhang, Sicong, Hui Yang, and Lisa Singh. 2014. "Increased Information Leakage from Text." *CEUR Workshop Proceedings* 1225 (November 2016): 41–42. doi:10.1023/A.
- Zhang, Tong, and Herbert H.P. Fang. 2000. "Digitization of DGGE (Denaturing Gradient Gel Electrophoresis) Profile and Cluster Analysis of Microbial Communities." *Biotechnology Letters* 22 (5): 399–405. doi:10.1023/A:1005680803442.
- Zhang, Zheng, Scott Schwartz, Lukas Wagner, and Webb Miller. 2000. "A Greedy Algorithm for Aligning DNA Sequences." *Journal of Computational Biology* 7 (1–2): 203–14. doi:10.1089/10665270050081478.
- Zhou, JunPei, YingQi Gu, ChangSong Zou, and MingHe Mo. 2007. "Phylogenetic Diversity of Bacteria in an Earth-Cave in Guizhou Province, Southwest of China." *Journal of Microbiology (Seoul, Korea)* 45 (2): 105–12. <http://www.ncbi.nlm.nih.gov/pubmed/17483794>.

6. Annex

6.1. Annex Table 1

Annex Table 1 -PCR-DGGE Bray-Curtis Similarity (group average) matrix data, REF – reference to each sample; Combining samples – The combination of different samples, based on their Ref, and the corresponding similarity.

Ref	Samples	Combining samples	Similarity (%)
1	k1	1+2 -> 16	73.27
2	k2	3+16 -> 17	66.84
3	k3	8+17 -> 18	63.58
4	k4	4+18 -> 19	57.02
5	k6	5+19 -> 20	55.15
6	k7	10+11 -> 21	52.94
7	k8	13+15 -> 22	39.80
8	k9	14+22 -> 23	35.30
9	k13	12+21 -> 24	34.06
10	k15	20+23 -> 25	31.32
11	k18	9+24 -> 26	29.03
12	k20	7+26 -> 27	27.33
13	k21	25+27 -> 28	24.10
14	k24	6+28 -> 29	15.19
15	k30		

6.2. Annex Table 2

Annex Table 2 - Pyrosequencing Bray-Curtis Similarity (group average) matrix data, REF –reference to each sample; Combining samples – The combination of different samples, based on their Ref, and the corresponding similarity.

Ref	Samples	Combining samples	Similarity (%)
1	k1	1+2 -> 14	87.89
2	k2	3+14 -> 15	64.85
3	k3	5+15 -> 16	56.69
4	k4	4+8 -> 17	53.52
5	k6	16+17 -> 18	44.04
6	k7	6+7 -> 19	36.89
7	k8	18+19 -> 20	22.28
8	k9	11+13 -> 21	16.18
9	k15	20+21 -> 22	14.09
10	k18	10+12 -> 23	9.38
11	k19	22+23 -> 24	5.46
12	k20	9+24 -> 25	0.78
13	k24		

6.3. Annex table 3

Annex Table 3 - Resume of most abundant OTUs distribution for every sample, and respective phylogenetic association. (OTUs with > 50sequences)

	K01	K02	K03	K04	K06	K07	K08	K09	K15	K18	K19	K20	K24	Phylogenetic association (from RDP)
OTU-1									x					Proteobacteria; Betaproteobacteria; Thiobacterales
OTU-2	x	x	x	x	x			x						Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
OTU-4	x	x	x	x				x						Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingorhabdus.
OTU-5													x	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium.
OTU-7												x		Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfuritalea
OTU-10			x		x									Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
OTU-11										x				Nitrospirae; Nitrospira; Nitrospirales; 4-29
OTU-12				x				x						Bacteroidetes; Sphingobacteriia; Sphingobacteriales
OTU-16										x				Proteobacteria; Gammaproteobacteria; Acidiferrobacterales; Acidiferrobacteraceae; Sulfurifustis
OTU-17							x							Proteobacteria; Betaproteobacteria; Neisseriales; Chromobacteriaceae; Vogesella
OTU-19					x									Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Limnohabitans
OTU-29								x						Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Arenimonas.
OTU-30										x				Nitrospirae; Nitrospira; Nitrospirales; 4-29
OTU-35										x				Proteobacteria; Alphaproteobacteria; BD7-3
OTU-40												x		Cyanobacteria; ML635J-21
OTU-91							x						x	Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas.
OTU-134									x					Proteobacteria; Betaproteobacteria; SBla14
OTU-1541												x		Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfuritalea
OTU-2109												x		Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Sulfuritalea
OTU-3070				x										Proteobacteria; Alphaproteobacteria; Sphingomonadales;