RESEARCH ARTICLE

Diversity and geochemical structuring of bacterial communities along a salinity gradient in a carbonate aquifer subject to seawater intrusion

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Abstract

In aquifers subject to saline water intrusion, the mixing zone between freshwater and saltwater displays strong physico-chemical gradients. Although the microbial component of these specific environments has been largely disregarded, the contribution of micro-organisms to biogeochemical reactions impacting water geochemistry has previously been conjectured. The objective of this study was to characterize and compare bacterial community diversity and composition along a vertical saline gradient in a carbonate coastal aquifer using high throughput sequencing of 16S rRNA genes. At different depths of the mixing zone, stable geochemical and hydrological conditions were associated with autochthonous bacterial communities harboring clearly distinct structures. Diversity pattern did not follow the salinity gradient, although multivariate analysis indicated that salinity was one of the major drivers of bacterial community composition, with organic carbon, pH and CO_2 partial pressure. Correlation analyses between the relative abundance of bacterial taxa and geochemical parameters suggested that rare taxa may contribute to biogeochemical processes taking place at the interface between freshwater and saltwater. Bacterial respiration or alternative metabolisms such as sulfide oxidation or organic acids production may be responsible for the acidification and the resulting induced calcite dissolution observed at a specific depth of the mixing zone.

Introduction

Groundwater accounts for almost 99% of the total volume of fresh water present on Earth (Younger, 2006). It is the world's main source of drinking water, providing up to 80% of drinking water in Europe (Struckmeier *et al.*, 2005). In the last 50 years the freshwater demand in the Mediterranean basin has doubled (Blinda & Thivet, 2009). As a result, massive freshwater withdrawals are observed in coastal aquifers. This situation, associated to climatic stress, such as low precipitation and warm temperature, often enhances the natural intrusion of saline water into the inland freshwater reservoirs. As a result, many coastal aquifers display severe and irreversible seawater intrusions that participate to the growing scarcity of the freshwater underground resources (Deyà Tortella & Tirado, 2011).

These groundwater–seawater interfaces, also called subterranean estuaries by analogy with surface estuaries, are characterized by important physico-chemical gradients in the mixing zone that favor geochemical reactions between the aquifer formation and the water mixture (Moore, 1999). For instance, in carbonated reservoirs, these reactions may include mineral precipitation or dissolution (Runnells, 1969), cation exchange (such as substitution of Ca^{2+} by Na^{+} introduced by seawater, Appelo & Postma, 2005) and redox processes, such as ferrous iron precipitation, which leads to the formation of 'iron curtain' deposits in sediments (Charette & Sholkovitz, 2002).

Although the microbial component of the subsurface has been largely disregarded for a long time, it is now widely accepted that microbial communities are the foundation of aquifer ecosystems, driving key bio-geochemical

FEMS MICROBIOLOGY ECOLOGY

cycles and representing the basis of the food web. The possible contribution of micro-organisms to the complex processes occurring in freshwater–seawater mixing zones (including those controlling calcite solubility) has often been conjectured (Smart *et al.*, 1988; Baceta *et al.*, 2001; Garing *et al.*, 2013) but never established because of the lack of knowledge concerning the microbial ecology of such specific environments (Hancock *et al.*, 2005).

Groundwater biota is heterogeneously distributed and characterized by strong environmental gradients. Furthermore, the different compartments of these subsurface ecosystems are often difficult to access. For these reasons, the understanding of the relationships between the biotic and abiotic components of aquifers remains elusive. Yet, deciphering how microbial communities are patterned in relation to environmental conditions represents an important challenge in microbial ecology (Stahl & Tiedje, 2002; Torsvik *et al.*, 2002). Major variations of physicochemical environmental conditions, such as salinity gradient, are generally associated with dramatic changes in community composition and richness for many microorganisms. Such changes often have consequences for the functioning of the whole ecosystem.

Few studies have addressed bacterial community structure in pristine groundwater and its relationships with geochemical and hydrochemical conditions. They mainly relied on molecular fingerprinting methods as t-RFLP (Griebler *et al.*, 2010; Stein *et al.*, 2010; Zhou *et al.*, 2012; Sirisena *et al.*, 2013) or denaturing gradient gel electrophoresis (Garrido *et al.*, 2014). The relatively low resolution of these techniques may result in an incomplete and biased estimation of the actual biodiversity and, more importantly, they do not provide any information about the taxonomic classification of the bacterial populations present. High throughput sequencing of bacterial 16S rRNA genes, characterized by a much higher resolution, was successfully applied to various complex environments (e.g. Roesch *et al.*, 2007; Galand *et al.*, 2009; Benson *et al.*, 2010; Caporaso *et al.*, 2011). This state-of-the-art technique represents a promising tool for providing a more detailed and accurate understanding of the bacterial diversity in groundwater.

The present study focuses on a coastal carbonate aquifer in Mallorca island (Spain), which displays a massive seawater intrusion into the southwest coast due to the excessive withdrawal of freshwater for touristic and agricultural activities. The geochemistry of this aquifer has been intensively monitored, highlighting calcite dissolution patterns and acidification of groundwater within the freshwater–saltwater mixing zone, the latter being possibly related to microbial respiration (Garing *et al.*, 2013).

The main objective of the present study is to characterize the diversity and taxonomic composition of the bacterial communities in this coastal aquifer, to determine how these communities are structured after the salinity gradient and whether autochthonous brackish bacterial communities could established in the mixing zone. To our knowledge, the present work represents the first investigation of the bacterial diversity along a vertical profile in the freshwater–saltwater mixing zone of a carbonate aquifer using a high throughput sequencing approach. The second goal is to establish the possible correlations between the groundwater bacterial community composition and the geochemical variables. Indeed, exploring the bacterial diversity is the first step towards the description of the complex biogeochemical processes occurring in the subsurface and particularly in the mixing zone.

Materials and methods

Mallorca experimental site

The study was conducted on the Llucmajor carbonate aquifer at an experimental site named Ses Sitjoles. The experimental site is located 6 km away from the coast in the southeast part of Mallorca island (Spain) and comprises a set of 100-m depth boreholes traversing the freshwater–saltwater interface. Previous studies detail the main geological and petrophysical properties of the Miocene reefal carbonate platform sampled at the Ses Sitjoles site (Jaeggi, 2006; Maria-Sube, 2008; Garing, 2011; Hebert, 2011; Garing *et al.*, 2013). A main feature of this aquifer is the presence of a saltwater intrusion that is currently intruding more than 10 km inland. At the site, the water table is located around 38 mbs (meter below the surface) and the freshwater becomes brackish around 62 mbs where the transition between the freshwater and the saltwater begins. This mixing zone extends to 80 mbs where the groundwater has salinities similar to seawater. The Ses Sitjoles site belongs to the French network of hydrogeological research sites ORE H+ (a national environmental research observatory). The geophysical and geochemical monitoring between 2003 and 2011 of the saltwater intrusion at the site was reported by Garing *et al.* (2013). It was shown that the geochemistry of the system was stable over the monitored period of time. However, a well-marked deviation of the water geochemistry from the theoretical conservative freshwater–saltwater mixing model was observed, suggesting the probable contribution of microbiological activity to complex biogeochemical processes.

Water sampling and chemical analysis

A sampling campaign was carried out in July 2011 and groundwater samples were collected in borehole MC2 at 51, 64, 71 and 77 mbs displaying 0.5%, 8%, 63% and 86% of saltwater end-member, respectively. Sampling was

performed using a multilevel groundwater monitoring system Westbay from Schlumberger Water Services (Black *et al.*, 1986). This equipment allows *in situ* sampling of the pore fluid at the formation pressure, thus guaranteeing the recovery of a sample which is representative of the groundwater at a given depth while avoiding the alteration of its physico-chemical properties during sampling. Groundwater sampling for microbiological analyses was performed after stabilization of chemical parameters. Six replicates of water samples (300 mL each) collected at the four depths in the borehole were immediately filtered through sterile 0.22-μm Nucleopore filters that were then transferred to a collection tube (Nunc), frozen in liquid nitrogen, and stored at −80 °C until DNA extraction.

The chemical composition of the water collected at 51 mbs in borehole MC2 matches the reference for freshwater found at this site. As illustrated in Fig. 1 and previously detailed in Garing *et al.* (2013), the mixing zone between freshwater and saltwater starts in the vicinity of 62 m depth and includes the groundwater sampled at 71 and 77 mbs. The conductivity measurements clearly highlight the salinity gradient formed along the aquifer, the concentrations of sodium, chlorine, magnesium and sulfate (Na^+, Cl^-, Mg^{2+}, SO_4^{2-}) following the same trend (Garing *et al.*, 2013). In the vicinity of 64 and 71 m depth, higher concentrations of organic carbon and depletion of O_2 coincide with water acidification and higher pCO_2 content. Among the geochemical parameters determined by Garing *et al.* (2013), the concentration of calcium (Ca^{2+}), Cl^-, and SO_4^{2-}, the total organic carbon content (TOC), the concentration of dissolved oxygen (DO), the pH, the saturation indices for calcite (SI_c) and dolomite (SI_d), and the CO_2 partial pressure [expressed as log(pCO_2) and calculated by geochemical numerical modeling using the Phreeq-C code, Parkhurst & Appelo, 1999] were used in the present study for statistical analyses.

DNA isolation

Three filters obtained from each depth (i.e. three replicates) were used to compare the efficiency of three DNA extraction kits (UltraClean Soil, Rapid Water, and Power Water DNA Isolation Kits; MoBio Laboratories Inc., Carlsbad, CA). DNA was then extracted from the remaining replicates with the extraction kit providing the highest DNA yields (Power Water DNA Isolation kit). For each depth, the bacterial community structure of three replicates were compared by a fingerprint method (Automated Ribosomal Intergenic Spacer Analysis) which confirmed the good reproducibility of sampling and the representativeness of each filter (data not shown). Because very low DNA concentrations were obtained for some DNA extracts, it was decided not to pool the DNA extracted from different filters, since it would have resulted in the dilution of the rare phylotypes and in the enrichment of the dominant ones. Pyrosequencing was performed on the most concentrated DNA extract obtained from each depth.

Pyrosequencing of bacterial 16S rRNA gene fragments

Universal primers 343F (5′-A/xxx/TACGGRAGGCAGCAG-3′) and 806R (5′-B/GGACTACCAGGGTATCTAAT-3′) were used to amplify a 463-bp region targeting the V3 and V4 variable regions of the bacterial 16S rRNA genes. A and B represent the two FLX Titanium adapters (A adapter sequence: 5′-CCATCTCATCCCTGCGTGTCTCCGAC-3′; B adapter sequence: 5′-CCTATCCCCTGTGTG

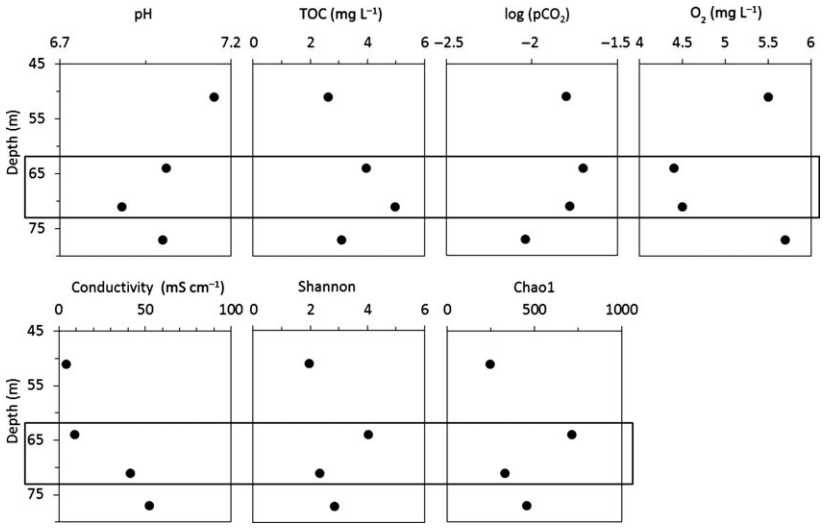

Fig. 1. Evolution of the main physico-chemical parameters (according to Garing *et al.*, 2013) and bacterial community diversity indexes with depth. The zone where the water geochemistry is inconsistent with theoretical conservative mixing of freshwater and seawater is indicated by the black frame.

CCTTGGCAGTC) and xxx represents the sample-specific bar-code sequence. PCR amplifications and pyrosequencing of the four amplicon libraries were performed on a Roche 454 Life Sciences Genome Sequencer FLX Titanium sequencer at Genoscreen (Lille, France).

Processing of pyrosequencing data and taxonomic classification

Data processing was conducted using the software program MOTHUR version 1.31 (Schloss *et al.*, 2009). Pyrosequencing flowgrams were filtered and denoised using the MOTHUR implementation of AMPLICONNOISE (Quince *et al.*, 2011) and sequences were removed from the analysis when they were < 200 bp, when they contained homopolymers longer than 8 bp, ambiguous bases, more than one mismatch to bar-code sequences or more than two mismatches to the forward primer sequence. We further removed sequences that did not align over the same span of nucleotide positions. Identical sequences were grouped and representative sequences were aligned against the SILVA bacterial and archaeal reference database using the Needleman–Wunsch algorithm (Needleman & Wunsch, 1970). Chimeric sequences were detected and removed using the implementation of CHIMERA UCHIME (Edgar *et al.*, 2011). A further screening step (pre-cluster) was applied to reduce sequencing noise by clustering reads differing by only one base every 100 bases (Huse *et al.*, 2010). The remaining high-quality reads were used to generate a distance matrix and clustering into operational taxonomic units (OTUs) defined at 97% cutoff using the average neighbor algorithm. Next, the OTUs were phylogenetically classified to the genus level using the naïve Bayesian classifier (80% confidence threshold) trained on the Ribosomal Database Project's taxonomic outline and implemented in MOTHUR with a confidence threshold of 80% and a manually modified SILVA bacterial 16S rRNA gene database. To obtain comparable data, the number of sequences per sample was made equal through random resampling (4891 sequences).

Statistical analysis

OTU-based diversity indices Shannon and Chao1 and rarefaction curves were calculated with MOTHUR at a level of 97% sequence similarity. To estimate community similarity among samples, a distance matrix was performed based on the Bray–Curtis dissimilarities of the OTU composition of the groundwater samples. The normalized abundances of the OTUs were square root-transformed before the analysis. Community relationships were visualized using principal coordinate analysis (PCoA) based on this distance matrix. To identify potential explanatory variables, environmental vector fitting was applied and projected to the PCoA ordination. In addition to pairwise comparison, Venn diagrams for graphical descriptions of unshared and shared OTUs between two, three or four samples were constructed. Pearson correlations were used to calculate the relationship between physico-chemical parameters and the relative abundance of bacterial taxa. All statistical analyses were carried out using the R statistical environment version 3.0.1 (R Development Core Team, 2012) using functions from the packages 'vegan' and 'stats' and custom R scripts.

Results

Bacterial community diversity and composition

A total of 43 355 sequence reads were generated in a single run of 454 pyrosequencing from the four independent 16S rRNA gene libraries. After trimming and processing with MOTHUR, 22 176 reads remained (284 bp average length). The number of sequences per sample was made equal through random resampling (4891 sequences) and clustering of the 19 564 remaining sequences led to the identification of 965 OTUs (including 457 singletons) defined at 97% identity. As determined by the Shannon and Chao1 indices, diversity and richness of the bacterial communities varied strongly in the vertical profile of the aquifer (Table 1). However, the variation of these two indices along depth did

Table 1. Number of operational taxonomic units (OTUs), estimated sample coverage and diversity indices obtained for the four water samples analyzed. Values in brackets are lower and upper 95% confidence intervals

Samples	No. Reads	Obs.OTUs*	Coverage†	Shannon‡	Chao1
51 mbs	4891	165	99	1.95 (1.88; 2.01)	248 (209; 321)
64 mbs	4891	472	96	4.02 (3.96; 4.08)	716 (644; 819)
71 mbs	4891	173	98	2.34 (2.29; 2.39)	333 (263; 457)
77 mbs	4891	265	97	2.85 (2.79; 2.90)	457 (386; 570)

*OTUs were defined at 97% cutoff.

†Coverage: sum of probabilities of observed classes calculated as [1 – (*n*/*N*)], where n is the number of singleton sequences and N is the total number of sequences.

‡Takes into account the number and evenness of species.

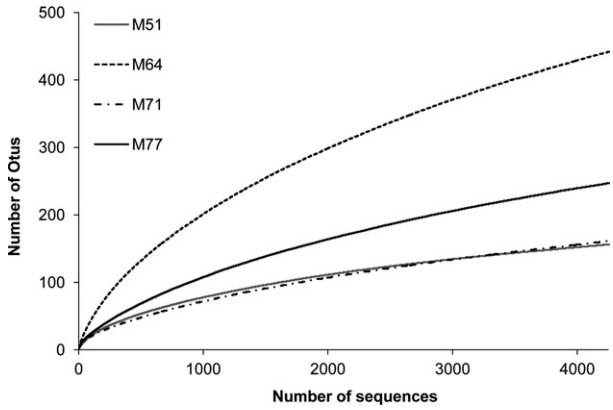

Fig. 2. Rarefaction curves of the bacterial 16S rRNA gene sequences from the four water samples based on operational taxonomic units (OTUs) calculated at 97% identity. The total number of sequences analyzed is plotted against the number of OTUs observed in the same community.

Table 2. Relative abundance (in %) of bacterial 16S rRNA gene sequences from each water sample assigned to different phyla

Taxa	Total*	51 mbs	64 mbs	71 mbs	77 mbs
Proteobacteria	80.1	91.1	77.5	93	58.8
Actinobacteria	1.3	3.4	0.6	< 0.1	1.0
Bacteroidetes	0.8	0.6	1.9	0.6	0.2
Firmicutes	0.2	0.1	0.5	< 0.1	< 0.1
Chlorobi	0.3	< 0.1	0.9	0	0.2
TM7	0.3	1.1	0	0	< 0.1
Chlamydiae	0.2	0.1	0.3	0.1	0.1
Verrucomicrobia	0.1	0	0.3	0	0
Acidobacteria	0.1	< 0.1	0.2	0	< 0.1
Gemmatimonadetes	0.1	< 0.1	0.2	< 0.1	0.1
OD1	0.1	0.2	< 0.1	0	0
Nitrospirae	< 0.1	0	0.1	0	0
Chloroflexi	< 0.1	0	0	0	< 0.1
Planctomycetes	< 0.1	0	< 0.1	0	0
WS3	< 0.1	0	< 0.1	0	0
Unclassified	16.6	3.3	17.3	6.2	39.5

*Calculated relatively to the total number of sequences retrieved in this study.

not follow the salinity profile (Fig. 1). The highest bacterial diversity and species richness were encountered at 64 mbs, at the beginning of the freshwater–saltwater mixing zone. This is in agreement with the rarefaction curves that tended to reach an asymptote for 51-, 71- and 77-mbs samples, but not for the 64-mbs sample (Fig. 2). Good's coverage, which provides an estimate of sampling completeness, ranged between 96% and 99% (Table 1).

Based on 16S rRNA gene sequences analysis, the bacterial phyla composing each community were identified and their relative abundance determined (Table 2). Seventeen percent of the total sequences could not be assigned to any known phylum, with the highest proportion of unassigned sequences obtained for 77 mbs (39.5%). In total, 15 bacterial phyla were identified across all the samples, with a majority of sequences assigned to *Proteobacteria* (58.8–93%), *Actinobacteria* (< 0.1–3.4%) and *Bacteroidetes* (0.2–1.9%). The 12 additional lineages were represented by less than 1% of the sequences (*Firmicutes, Chlorobi,* TM7, *Chlamydiae*, *Verrucomicrobia*, *Acidobacteria*, *Gemmatimonadetes*, OD1, *Nitrospirae*, *Chloroflexi*, *Planctomycetes*, and WS3).

Analysis of the phylum *Proteobacteria* at the class level showed that all samples except 77 mbs were dominated by *Alphaproteobacteria* comprising 81%, 46% and 53% of the total sequences at 51, 64 and 71 mbs, respectively (Fig. 3). The bacterial community at 64 mbs was characterized by a relatively high proportion of *Betaproteobacteria* (21% of the total sequences) compared with the other depths, where this class represented no more than 2% of the total sequences. The *Gammaproteobacteria*, which were under-represented in the 51- and 64-mbs bacterial communities (4% of the total sequences), represented 36% and 37% of the total community at 71 and 77 mbs, respectively. The *Deltaproteobacteria* were identified in low proportions at 51, 64 and 77 mbs. The rare taxa *Zeta-* and *Epsilonproteobacteria* were only detected at 71 and 77 m depth, respectively, where they represented less than 1% of the total sequences.

Bacterial communities along the freshwater–saltwater mixing zone

Bacterial communities were compared based on the community membership in order to explore the extent of spatial variation of bacterial community patterns along the mixing zone. Figure 4 depicts a Venn diagram representing the unique and overlapping OTUs in the four

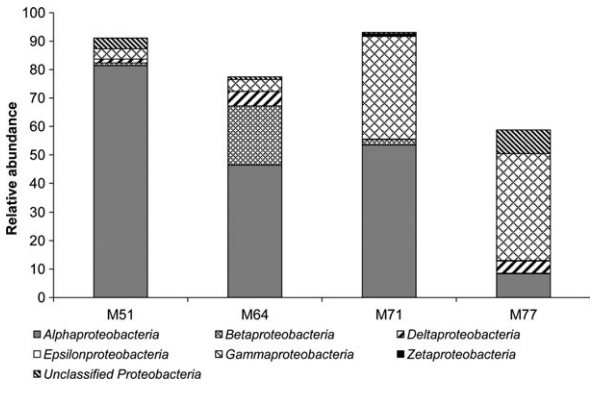

Fig. 3. Distribution of *Proteobacteria* classes identified in the four bacterial communites.

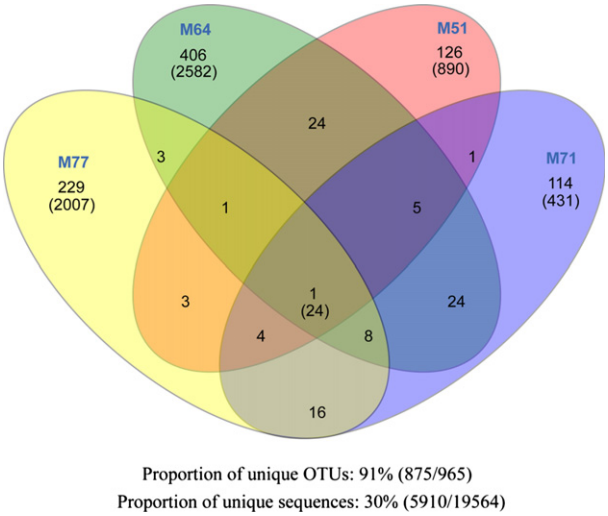

Fig. 4. Venn diagram showing the number of shared and unique operational taxonomic units (OTUs) between the bacterial communities characterized at the four depths of the aquifer. The number of sequences associated with OTUs is shown in parentheses. OTUs are defined by 97% sequence similarity.

communities analyzed. Across the 965 OTUs retrieved from the four groundwater samples, a relatively high percentage of OTU in each sample were unique (77%, 86%, 68% and 87%, respectively, for 51, 64, 71 and 77 mbs). Only one OTU, corresponding to 24 sequences, was shared between the four samples. These results indicated that very distinct bacterial communities inhabit the different depths of the freshwater–saltwater mixing zone. Moreover, although the percentage of shared OTUs between the four bacterial communities was very low (9% shared between two or more samples), the majority of the total sequences (70%) were shared between at least two depths (Fig. 4). Dominant OTUs are more widely distributed along the aquifer compared with minority taxa, which appeared more specific to one or two depths.

These significant shifts in bacterial composition along the mixing zone are also reflected by pronounced variations at the family level (Fig. 5). Among the *Alphaproteobacteria*, distinct families were dominant at 51, 64 and 71 mbs (the *Caulobacteraceae*, *Rhodocyclaceae* and *Hyphomonadaceae*, respectively). The deepest water (77 mbs) was mostly characterized by the abundance of unclassified *Gammaproteobacteria* and *Alteromonadaceae*. In the water collected at 64 mbs, the *Betaproteobacteria* were mainly composed of members of the *Rhodocyclaceae* family (genera *Thauera*, *Azoarcus* and *Denitromonas*).

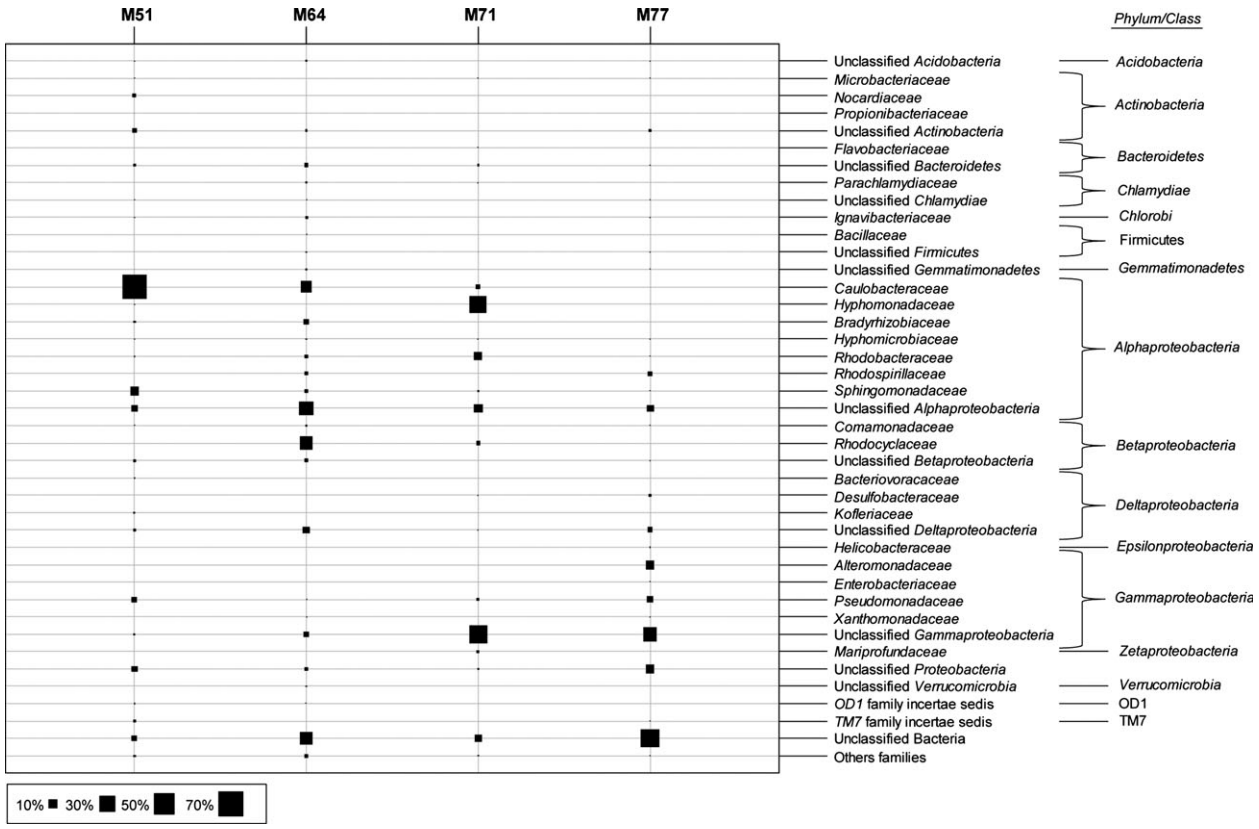

Fig. 5. Pyrosequencing profiles showing the relative abundance of bacterial taxa (family level) over the aquifer depth.

Bacterial community structure and relationship with environmental parameters

PCoA ordination of the dissimilarity matrix of the relative abundance of OTUs demonstrated that the bacterial community composition varied with depth (Fig. 6). The first dimension of PCoA separated the 77-mbs sample from the other three samples and accounted for 40% of the community variability. The 51-, 64- and 71-mbs samples were mainly separated along the second axis, accounting for an additional 33% of the community variability. Vector fitting of possible explanatory environmental variables highlighted Cl^-, TOC, pH and log(pCO_2) as the most powerful predictors separating bacterial communities along the mixing zone ($P < 0.05$, Table 3, Fig. 6). According to fitting analyses, high values of CO_2 partial pressure [log(pCO_2)] were correlated to the bacterial community structure at 64 mbs, whereas the low value of this factor explained the composition of the bacterial community at 77 m depth. Higher salinity, reflected by higher concentrations of Cl^-, was the main environmental factor shaping the structure of the bacterial community at 71 and 77 m depth. Bacterial community structure at 51 m was shaped mainly by a relatively high pH and a relatively low TOC, whereas at 71 m the opposite trend was observed (Fig. 6).

Pearson correlation tests were carried out to identify potential co-variation between physico-chemical parameters and the abundance of bacterial groups at different taxonomic levels. Only the statistically significant relationships (Bonferroni corrected; $P < 0.05$) obtained at the class and order levels are summarized in Table 4 for taxa representing a total of five sequences or more. The relative abundance of *Gammaproteobacteria* was positively correlated with salinity (i.e. concentrations of Cl^-, and consequently of SO_4^{2-} and Ca^{2+}) and the saturation index of dolomite (SI_d). *Epsilonproteobacteria* abundance was negatively correlated with log(pCO_2) whereas *Alphaproteobacteria* abundance was positively correlated with the saturation index of calcite (SI_c). At the order level, the *Kordiimonadales* were positively correlated to the TOC, the *Burkholderiales* to the pH, the *Pseudomonadales* to the O_2 and uncultured *Gammaproteobacteria* to the Ca^{2+}. Four bacterial orders were negatively correlated to log(pCO_2): *Desulfobacterales*, *Desulfovibrionales*, *Campylobacterales*, and *Alteromonadales* (Table 4).

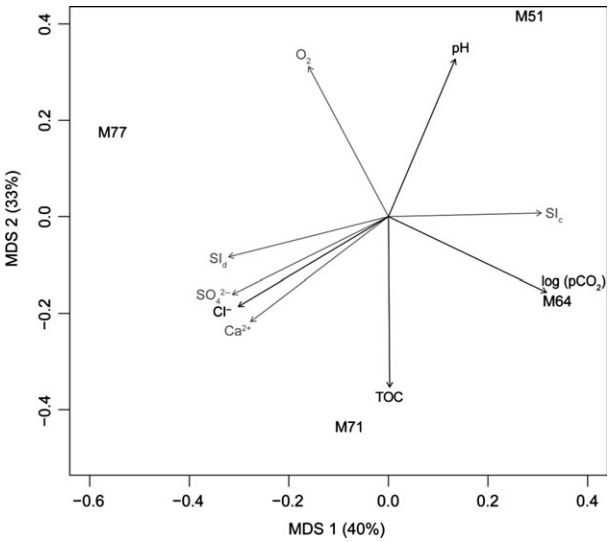

Fig. 6. Principal coordinate analysis (PCoA) of the bacterial community matrix (based on operational taxonomic units) and vector fitting of the environmental variables. The variation explained by the axes is indicated. Gray arrows and black arrows represent non-significant and significant fittings, respectively (Bonferroni-corrected, $P < 0.05$). The corresponding r^2 correlation coefficients and *P*-values are listed in Table 3.

Discussion

The main objectives of this study were the in-depth characterization of the bacterial diversity and community composition in groundwater subject to a saltwater intrusion and the establishment of possible relationships

Table 3. Main physico-chemical parameters (mg L^{-1}) of the water sampled at the four depths. r^2 – correlation coefficient and corresponding *P*-value indicating goodness of fit and significance determined by vector fitting to the PCoA (bold font with $*P < 0.05$)

					Vector fit	
Water depth	51 mbs	64 mbs	71 mbs	77 mbs	r^2	*P*
Ca^{2+}	154	255	843	736	0.99	0.224
Cl^-	1080	2790	15 550	20 930	**1.00**	**0.001***
SO_4^{2-}	282	440	2280	2940	0.99	0.118
TOC	2.63	3.96	4.97	3.09	**0.99**	**0.001***
O_2	5.5	4.4	4.5	5.7	0.98	0.163
pH	7.15	7.01	6.88	7	**0.99**	**0.034***
SI_c	−0.02	−0.07	−0.06	−0.15	0.75	0.435
SI_d	0.21	0.16	0.39	0.42	0.88	0.518
Log(pCO_2)	−1.8	−1.7	−1.78	−2.04	**0.99**	**0.038***

Table 4. Environmental factors associated with variations of the bacterial community structure at the class or order level in the four water samples. Pearson's correlation coefficient (r) indicates correlations between taxa relative abundance and the water main physico-chemical parameters. Only taxa representing five or more sequences and with at least one statistically significant correlation coefficient are shown. Correlation coefficients in bold font with * are statistically significant (Bonferroni corrected; $P < 0.05$)

Taxonomic level	Taxa name	Number of sequences					Pearson's coefficient correlation (r) with main environmental parameters							
		Total	51 m	64 m	71 m	77 m	Ca^{2+}	Cl^-	SO_4^{2-}	TOC	pH	O_2	SI_c	SI_d	$Log(pCO_2)$
Class	*Alphaproteobacteria*	9277	3981	2271	2616	409	−0.602	−0.788	−0.766	−0.054	0.437	−0.211	**0.993***	−0.605	0.71
	Epsilonproteobacteria	10	0	0	0	10	0	0.746	0.73	−0.37	−0.06	0.671	−0.918	0.645	**−0.956***
	Gammaproteobacteria	3977	177	204	1766	1830	**0.982***	**0.978***	**0.984***	0.397	−0.725	0.142	−0.659	**0.983***	−0.647
Order	*Bacillales*	10	4	6	0	0	−0.915	−0.915	−0.928	−0.254	0.563	−0.307	0.510	**−0.989***	0.683
	Kordiimonadales	5	0	2	3	0	0.358	0.041	0.049	**0.973***	−0.785	−0.937	0.224	0.026	0.622
	Burkholderiales	76	46	20	1	9	−0.897	−0.802	−0.798	−0.748	**0.957***	0.378	0.615	−0.709	0.248
	Desulfobacterales	52	0	1	2	49	0.492	0.766	0.751	−0.338	−0.094	0.650	−0.928	0.664	**−0.953***
	Desulfovibrionales	6	0	0	1	5	0.629	0.862	0.851	−0.211	−0.228	0.595	−0.926	0.780	**−0.956***
	Campylobacterales	10	0	0	0	10	0.465	0.746	0.730	−0.370	−0.060	0.671	−0.918	0.645	**−0.956***
	Alteromonadales	446	0	1	0	445	0.464	0.745	0.730	−0.370	−0.060	0.671	−0.919	0.644	**−0.955***
	Pseudomonadales	450	157	6	48	239	0.170	0.454	0.453	−0.716	0.381	**0.974***	−0.495	0.495	−0.911
	Unc. *Gammaproteobacteria*	1438	1	5	1432	0	**0.989***	0.877	0.888	0.638	−0.864	−0.127	−0.460	0.905	−0.388

between bacterial community composition and geochemical parameters.

Bacterial diversity and taxonomic composition along the aquifer

The present study focused on the freshwater–saltwater mixing zone in a carbonate coastal aquifer, characterized by a particular geochemical signature, where complex biogeochemical processes are thought to occur. Bacterial diversity, expressed by the Shannon index, varies greatly along the vertical profile (Table 1, Fig. 1). At 51, 71 and 77 mbs, the bacterial diversity is in agreement with what is generally observed in uncontaminated groundwaters (Griebler *et al.*, 2010; Zhou *et al.*, 2012; Sirisena *et al.*, 2013). The highest diversity (Shannon = 4.02) was observed at 64 mbs (Cl^- = 2790 mg L^{-1}) at the beginning of the groundwater-saltwater mixing zone. More than 50% of the 16S rRNA gene sequences characterizing the 64-mbs water sample were not detected at the other depths (Fig. 4). The deeper (77 mbs) and more saline water (Cl^- = 20 930 mg L^{-1}) exhibited higher diversity than waters sampled at 51 and 71 mbs. Contrary to what was observed in the Delaware Bay estuary (Campbell & Kirchman, 2013), the establishment of brackish conditions in the vertical subterranean estuary studied did not result in a decrease in bacterial diversity. However, our results are in agreement with what was observed in the rather stable estuary of the Baltic Sea, where no marked change of Shannon diversity index occurred along the salinity gradient (Herlemann *et al.*, 2011). The diversity indices did not display significant correlation to any of the measured physico-chemical variables (data not shown), thus the parameters explaining the trend observed for the diversity remain to be elucidated (a combination of several parameters may explain this pattern). Nonetheless, the results showed that an increase in salinity is not associated to a decrease in bacterial diversity.

Consistent with previous cultivation-independent studies (Lopez-Archilla *et al.*, 2007; Griebler & Lueders, 2009), our results revealed groundwater bacterial communities dominated by *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, with other phyla detected in lower proportions such as *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Nitrospirae*, *Planctomycetes*, WS3 and TM7 candidate divisions. The pyrosequencing approach conducted in the present study allowed the additional identification of 'rare' bacterial phyla (representing less than 1% of the sequences) which, to our knowledge, have not been detected before in groundwater: *Chlorobi*, *Chlamydiae*, *Gemmatimonadetes* and OD1. Unexpectedly, *Alphaproteobacteria* were dominant at 51, 64 and 71 m depth, whereas *Betaproteobacteria* are generally found to be the

most abundant group in groundwaters (Pedersen *et al.*, 1996; Miyoshi *et al.*, 2005; Lopez-Archilla *et al.*, 2007; Zhou *et al.*, 2012). An increase of the relative abundance of *Gammaproteobacteria* with increasing salinity was observed, in agreement with previous studies (e.g. Lopez-Archilla *et al.*, 2007). More generally, the abundance of bacteria distinctive of marine environments increased along the salinity gradient, as reported in an aerial estuary (Herlemann *et al.*, 2011).

The more diverse groundwater community (64 mbs) was characterized by the presence of *Betaproteobacteria* belonging to genera *Thauera* and *Azoarcus*, which were not detected in the other depths. *Thauera* spp. are known for their versatile metabolism (both heterotrophic and chemolithoautotrophic) and their capacity to degrade aromatic hydrocarbons and to denitrify (Liu *et al.*, 2013). *Azoarcus* spp. are nitrogen-fixating bacteria, usually found in contaminated water, and also include denitrifiers. Numerous other bacterial taxa were detected only at 64 m depth, including rare phylotypes such as the autotrophic nitrite-oxidizing *Nitrospira* spp.

At 71 mbs, the bacterial community was characterized by the abundance of sequences related to *Hyphomonas* spp., a heterotrophic *Alphaproteobacteria* isolated from a wide range of aquatic environments, including freshwater and seawater. These bacteria are able to use a large range of substrates and play an important role in biofilm formation by producing extracellular polymeric substances (Quintero *et al.*, 1998). *Rhodobacteraceae,* which were represented much more at 71 m depth than in the rest of the water column, are also known for their capacity to form biofilms (Elifantz *et al.*, 2013). Although they represent key members of marine communities, *Rhodobacteraceae* were previously detected in groundwater (Lopez-Archilla *et al.*, 2007) and in artesian spring water (Ball & Crawford, 2006). Among the rare phylotypes detected specifically at 71 mbs were bacteria related to *Mariprofondus* sp., an obligate chemolithoautotrophic iron-oxidizing bacterium. Their presence coincides with the occurrence of patchy iron oxide deposits on the aquifer rock at the vicinity of 71 mbs (data not shown). Furthermore, the specific presence of bacteria related to *Mariprofondus* sp. in the lower pH and higher TOC zone of the aquifer, where the concentration in calcium is higher than predicted, is also in agreement with a study showing that *Marioprondus ferroxidans* could lower the pH by producing organic compounds and thus contribute to the release of Ca^{2+} from a mineral surface in a culture experiment (Bennett *et al.*, 2014). *Mariprofondus* spp. are the only representative of the recently described *Zetaproteobacteria* class (Emerson *et al.*, 2007). Despite their apparent wide distribution and biogeochemical importance, knowledge about the environmental significance of *Zetaproteobacteria* remains limited.

In the deeper water (77 mbs) almost 40% of the sequences could not be related to any known bacteria (Table 2). The dominant OTUs that could be classified belonged to the *Gammaproteobacteria* and include *Alteromonadales* (related to *Microbulbifer* spp.), *Pseudomonadales* (related to *Pseudomonas* spp.) and unclassified *Gammaproteobacteria*. Whereas *Alteromonadales* are primarily marine inhabitants, *Pseudomonodales* can be found in a wide range of environments, including freshwater and saltwater. *Pseudomonas* spp. are particularly well adapted for the conditions encountered in aquifers because of their flexibility in terms of the organic substrates and the electron acceptors they can use (Chapelle, 2000). The more abundant OTU among the unclassified *Gammaproteobacteria* was analyzed by BLAST and could be related to *Thioprofundum* spp., a chemolithoautotrophic, sulfur-oxidizing denitrifier, moderately halophilic genus, isolated from marine and saline environments (Takai *et al.*, 2009; Mori *et al.*, 2011). Another group of chemolithoautotrophic sulfur-oxidizing bacteria was specifically detected at 77 mbs: *Sulfurimonas* spp., belonging to the *Epsilonproteobacteria*. Their potential ecological niches include brackish and marine environments, where they play a significant role in sulfur-dependent biogeochemical cycles (Labrenz *et al.*, 2013).

Autochthonous brackish communities

The brackish bacterial communities characterized at 64, 71 and 77 mbs showed marked differences in their diversity and composition compared with their freshwater counterpart (at 51 mbs). Compared with surface waters that are exposed to important diel and seasonal dynamics, groundwaters are rather stable environments where autochthonous microbial communities are likely to establish (Farnleitner *et al.*, 2005). Groundwater habitats are characterized by a vertical layering, specific to every aquifer, which relies on hydrological, physico-chemical and geological heterogeneity (Griebler & Lueders, 2009). In coastal aquifers subject to saltwater intrusion, this stratification is exacerbated by the formation of a vertical physico-chemical gradient in the mixing zone. Long-term monitoring of the aquifer highlighted the stability of the physico-chemical conditions over time (Garing *et al.*, 2013). The long water residence time and the stable hydrological conditions in the aquifer proved to promote the establishment of autochthonous bacterial communities. Indeed, site-specific bacterial communities characterized by very distinct structures were highlighted in the four depths. The more diverse communities encountered in the mixing zone (at 64 and 71 mbs) harbored respectively 53% and 41% of sequences that were not detected in another depth; and only one rare OTU (24 sequences)

was common to the four samples. These findings indicate a strong layering of the bacterial communities across the depth and suggest very limited vertical groundwater flux.

Main physico-chemical parameters shaping the bacterial community structure

So far investigations of the structure of aquatic microbial communities at freshwater–saltwater interfaces have been limited to surface aquatic environments, where salinity was suggested to be the major determinant of microbial community composition (Wu *et al.*, 2006; Lozupone & Knight, 2007; Fortunato *et al.*, 2012). In the present study, based on fitting analysis, salinity also appeared to be one of the main environmental variables shaping the groundwater bacterial community composition within the coastal aquifer mixing zone (Table 3, Fig. 6). In addition to salinity, the other environmental factors significantly correlated to bacterial community structure in this subterranean estuary were pH, TOC and log(pCO_2). pH has been previously identified as a major driver of microbial communities in numerous surface ecosystems (e.g. Fierer *et al.*, 2007 and references therein; Lauber *et al.*, 2009; Kuramae *et al.*, 2012). In groundwater, organic matter is another important driving factor of the distribution and activity of micro-organisms (Goldscheider *et al.*, 2006; Griebler & Lueders, 2009). Organic carbon contents in the studied aquifer were within the range of concentrations generally observed in pristine groundwaters (Gooddy & Hinsby, 2008). At 71 m depth, and to a lesser extent at 64 m depth, an increase in organic carbon content (up to 5 mg L^{-1}) was observed. The accumulation of organic carbon along a density gradient was previously described at the freshwater–saltwater interface of the highly stratified Krka estuary (Zutic & Legovic, 1987). Determination of the nature and origin of the organic matter accumulated in the studied aquifer would require further investigations. Yet, this organic carbon represents a potential food source for heterotrophic micro-organisms that may favor the development of organotrophic bacteria such as *Hyphomas* spp. and *Kordiimonadales* identified at 71 m depth. Although the order *Kordiimonadales* was only represented by five sequences, its relative abundance in the aquifer was significantly positively correlated with TOC content (Table 4).

Microbial processes that may affect groundwater geochemistry

Diverse metabolically versatile bacteria were identified along the mixing zone of the studied coastal aquifer, including chemolithoautotrophs and heterotrophs, bacteria involved in sulfur, nitrogen and iron cycling. Thus, the autochthonous bacterial communities have the potential to facilitate a wide range of metabolic and biogeochemical processes in this system.

In the vicinity of 71 mbs, the maximum concentration of organic carbon and depletion of O_2 coincided with the lowest pH values and highest pCO_2 measured along the aquifer (Fig. 1; Garing *et al.*, 2013). The presence of diverse heterotrophic bacteria corroborates the previous conjecture that bacterial respiration, consuming O_2 and releasing CO_2, can explain the acidification and the resulting induced calcite dissolution (Smart *et al.*, 1988; Garing *et al.*, 2013). Based on the results of this study, alternative bacterial metabolisms potentially responsible for an acidification at 71 mbs include sulfide oxidation (production of sulfuric acid) by sulfur oxidizers or production of organic acids by *Mariprofondus* spp.

The coexistence of sulfur-oxidizers and sulfate-reducing bacteria in the aquifer indicates the possible active sulfur cycling in this groundwater ecosystem. Similarly, and even if nitrate concentrations were below the detection limit (data not shown) in this rather oxygenated groundwater, functional groups involved in nitrogen cycling were identified (denitrifiers, nitrite-oxidizers and N_2 fixing bacteria).

Finally, several bacterial groups known to have the capacity to enhance dolomitization were detected at 77 m depth, including sulfate-reducing and sulfur-oxidizing bacteria (Vasconcelos *et al.*, 1995; Moreira *et al.*, 2004). However, their precise role in the dolomitization process is not clear and needs to be elucidated. Indeed, no dolomite was observed around 77 m depth, in spite of favorable conditions.

Conclusion

In the mixing zone between freshwater and saltwater, the strong physico-chemical gradient triggered marked changes in the bacterial community composition and diversity. Stable hydrological and physico-chemical conditions were conducive to the establishment of autochthonous bacterial communities. If salinity appears to be the main factor shaping the structure of these communities, it does not drive their diversity. TOC, pH and partial pressure of CO_2 also influenced the structure of the bacterial community. Both metabolically versatile and specialized bacteria harboring a wide range of metabolic capacities were identified at the different depths of the aquifer. Furthermore, statistical analyses revealed significant correlations between the relative abundance of specific bacterial taxa and environmental variables. These findings corroborate the probable role these micro-organisms play in diverse biogeochemical processes, leading to modifications of groundwater and mineral geochemistry.

The acidification observed in the vicinity of 71 mbs can be attributed to the production of organic acids by bacteria related to *Marioprofondus* or to heterotrophic respiration process stimulated by the accumulation of organic carbon.

This work represents the first in-depth characterization of bacterial community composition and diversity along a freshwater–saltwater mixing zone in an aquifer. Precise ecological interpretations are limited, mainly because (1) abundance and activity are not necessarily correlated and the actual contribution of rare taxa to biogeochemical processes remains to be determined (Campbell *et al.*, 2011); and (2) groundwater only partially reflects the bacterial communities thriving in subsurface environments due to the significant proportion of bacteria attached to the rock surface (Griebler & Lueders, 2009 and reference therein).

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