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Diversity and spatial distribution of metal-reducing bacterial assemblages in groundwaters of different redox conditions

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Summary. The spatial distribution and diversity of metal-reducing bacterial assemblages belonging to *Geobacteraceae* were studied in groundwaters with different physicochemical characteristics by means of terminal-restriction fragment length polymorphism (T-RFLP) molecular fingerprinting, as applied to the 16S rRNA gene. The physicochemical conditions of these environments were unfavorable to support active-metal-reducing processes. The highest diversity of *Geobacteraceae* was observed in groundwater samples characterized by the highest dissolved Fe and Mn concentrations. T-RFLP analyses revealed major differences in the *Geobacteraceae* ribotype diversity and community composition of the groundwater samples as well as a considerable variability and spatial turnover of *Geobacteraceae* assemblages. Results from this work suggest that changes in the physicochemical characteristics of the aquifer deeply influence the richness and community structure of *Geobacteraceae*, even in those systems in which metal-reduction processes are not dominant. [**Int Microbiol** 2009; 12(3):153-159]

Keywords: *Geobacteraceae* · metal-reducing bacteria · manganese-iron in aquifers · groundwater

Introduction

Reduction/oxidation (redox) processes deeply affect the chemical quality of groundwater in aquifer systems [26]. Redox processes, indeed, can mobilize or immobilize toxic metals associated with naturally occurring aquifer materials [38], contribute to the degradation or preservation of anthropogenic contaminants [3], and generate unwanted by-products such as dissolved ferrous iron (Fe^{2+}), hydrogen sulfide (H_2S), and methane (CH_4) [6]. Iron (Fe) and manganese (Mn) are ubiquitous transitional metals in soil and sediment, where they occur predominantly as solid-phase minerals in oxidized forms [10]. The cycling of these elements in subsurface environments is largely dependent upon reduction processes mediated by prokaryotes (i.e., dissimilatory reduction), which transform the insoluble oxidized forms [i.e., Fe(III) and Mn(III, IV)] into soluble reduced forms [i.e., Fe(II) and Mn(II)] [19]. Prokaryotes able to use oxidized metals as terminal electron acceptors to generate energy for biosynthesis and cell maintenance comprise many different taxonomic groups and include a large variety of *Bacteria* (among them, members of the family *Geobacteraceae* and several phylotypes belonging to the sulfate-reducing bacteria cluster [8,11]) and *Archaea* [19,40], both of which carry out different mechanisms of metal reduction, such as extracellular electron trans-

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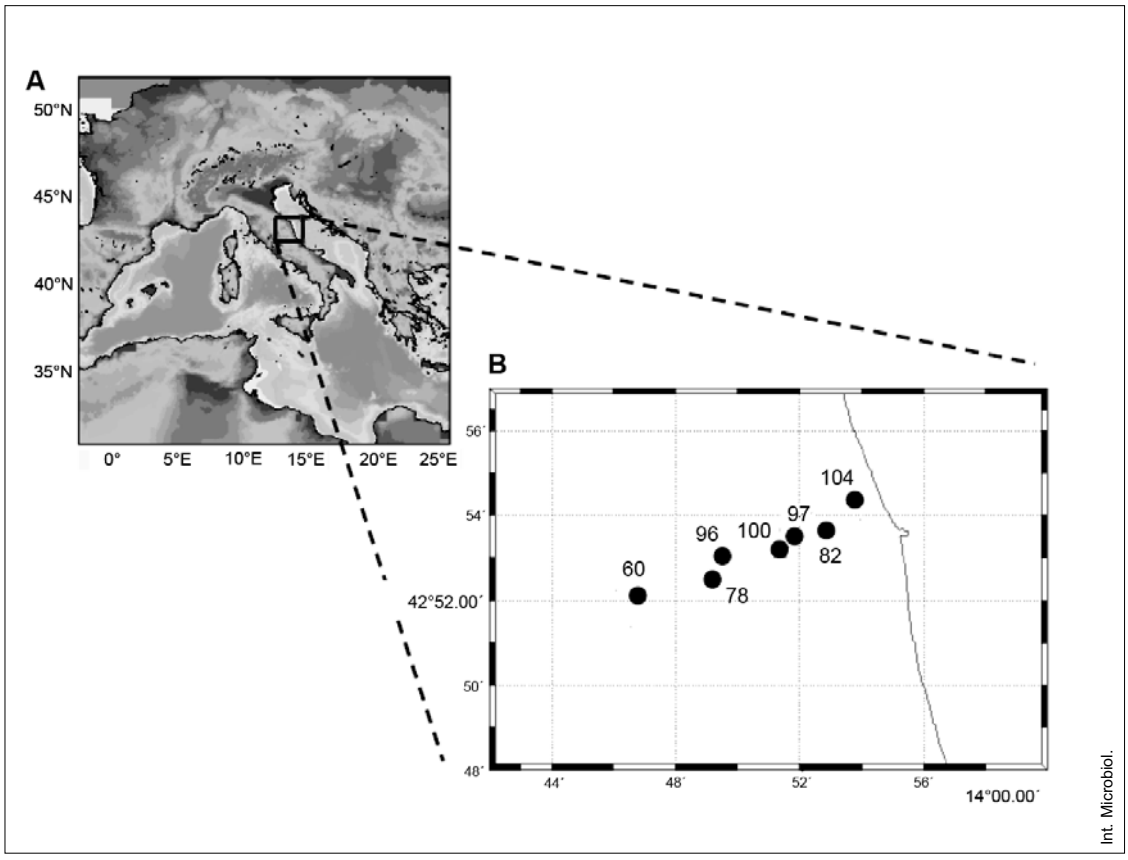

Fig. 1. Location of the studied aquifer system (**A**), showing the positions of the sampled wells (**B**).

fer requiring direct cell-oxide contact and the production of soluble metal chelators and electron shuttling compounds (e.g., quinones) [5,28,29]. These mechanisms influence the extent and rates of iron and manganese reduction in the subsurface and thus have a major impact on the geochemical characteristics of groundwater [19].

Previous studies on metal-reducing bacteria in subsurface environments showed that the *Geobacteraceae* community composition is influenced, to a great extent, by the physicochemical traits of the aquifer [18,35]. Ludvigsen et al. [20] pointed out that several redox processes, such as methanogenesis, sulfate-reduction, and iron-reduction, can take place simultaneously within the same aquifer. Rooney-Varga et al. [36] reported that *Geobacter* spp. play an important role in the anaerobic oxidation of aromatic hydrocarbons in contaminated aquifers. Together, these findings indicate the complex nature of the interactions between metal-reducing bacterial assemblages and the physicochemical characteristics of subsurface environments. In this work, we studied the diversity and spatial distribution of metal-reducing bacterial assemblages belonging to *Geobacteraceae* in groundwater samples displaying different physicochemical characteristics.

Materials and methods

Sampling. Groundwater samples were collected from an aquifer system (Marche region, Central Italy) (Fig. 1A) having a groundwater level at ca. 10–12 m below the ground surface. Samples were collected from seven wells (numbered 60, 78, 82, 96, 97, 100, and 104) in an area of ca. 15 km^2 (Fig. 1B). The aquifer is characterized by high concentrations of dissolved manganese, iron, and organic compounds typical of a large industrial site [http://www.arpa.marche.it]. Well number 60 was located upstream from the putative source of contamination, and all other wells downstream of the aquifer flow path. Before the water was collected, the wells were purged under controlled flow until the temperature, electrical conductivity, and pH levels were stabilized (3–5 volumes) [30]. Water samples were transferred into sterile polyethylene bottles previously rinsed with HCl (0.1 N) and stored at 4°C in the dark. For Fe and Mn determinations, samples were immediately filtered and acidified. Based on this protocol, it was assumed that the iron and manganese measurements were the end-products of dissimilatory Fe(III) and Mn(IV) reduction (i.e., Fe(II) and Mn(II); [19]).

Bottom sediments were collected from five wells (78, 82, 96, 97, and 104), transferred into sterile Falcon test tubes, and stored at 4°C in the dark. For chemical analyses, samples were transferred into glass bottles and stored at 4°C. For total prokaryotic counts, an aliquot of each sample was fixed as described [22,31], with three replicates per sample. For molecular analyses, 2 l of water from each aquifer was filtered onto Nuclepore polycarbonate filters (47 mm diameter, 0.2-µm pore size) and the filters stored at –20°C. For molecular analyses of sediment samples, the sediments were placed into a sterile test tube and stored at –20°C.

Table 1. Main physicochemical variables of the studied groundwaters

Well	Conductivity (µS/cm)	Temperature (°C)	pH	Oxygen (mg/l)	Nitrates (mg/l)	Sulfates (mg/l)	Iron (µg/l)	Manganese (µg/l)
60	1026	17.2	6.68	4.0	141	147	21	17
78	1107	15.7	7.27	0.3	133	193	50	100
82	928	16.5	7.55	0.2	4	58	280	534
96	1390	15.0	6.87	3.9	126	213	47	211
97	1830	15.6	7.33	0.2	152	319	29	99
100	1320	16.1	7.02	0.3	41	202	48	240
104	670	15.5	6.15	0.2	19	49	34	100

Environmental variables. A portable pH-meter (model HD 8705; Delta Ohm, Italy) was used to measure pH, nitrates, sulfates, iron, and total manganese concentrations were determined according to standard methods [1]. Dissolved oxygen concentrations, conductivity, and temperature were measured in situ using a CTD probe SBE 19 (Sea Bird Electronics).

Total prokaryotic abundance. Total abundance of prokaryotes in water and sediment samples was determined by epifluorescence microscopy (Zeiss Axioskop 2, 1000×). For water samples, an aliquot of each fixed sample was filtered onto 0.2-µm pore size Anodisc filters, stained with 20 µl of SYBR Green I (diluted 1:20), washed twice with sterile Milli-Q water, and mounted onto microscopic slides [31]. For sediments, the fixed samples were treated with pyrophosphate, sonicated, properly diluted, and stained with acridine orange as described [22]. These samples were then filtered onto 0.2-µm pore size, black-stained Nuclepore filters, washed twice with sterile Milli-Q water, and mounted onto microscopic slides. At least 400 cells were counted for each slide. Total counts of prokaryotes were expressed as cells/l (for water samples) or cells/g dry weight (for sediment samples after desiccation, 60°C, 48 h).

Molecular analyses. T-RFLP was used to analyze the 16S rRNA gene in studies of the phylogenetic diversity, community composition, and spatial distribution patterns of *Geobacteraceae* [18,21]. DNA was extracted from water and sediment using the UltraClean soil DNA isolation kit (MoBio Laboratoires, USA). For water samples, the kit was adapted as described [39]; for sediment samples, 1 g of sediment was processed. DNA concentrations were determined fluorometrically [9] and 5 ng of DNA was used for PCR amplifications. The quality/purity from the inhibitors was checked by amplifying the 16S rRNA gene using the universal primers 27F and 907R [21]. All DNA extracts were successfully amplified. To amplify the 16S rRNA genes of metal-reducing bacterial assemblages, the primer sets Geobact494F (HEX-labeled) and Geobact825R, specific for the 16S rDNA of *Geobacteraceae*, were used [16,36]. All PCRs were carried out using the MasterTaq kit (Eppendorf) and the conditions described by Holmes et al. [16]. The PCR products were checked on an agarose-TBE gel (1%). Two PCRs were run for each sample and the products then pooled to minimize biases [32,33]. This process was carried out in duplicate, for a total of four different PCRs for each sample. Purified 16S rDNA amplicons (100 ng) from each of the two duplicate PCR were digested separately with 10 U of the enzyme *Rsa*I (Promega) at 37°C for 3 h [21]. Fragments were analyzed in GeneScan mode in an ABI Prism 3100 Genetic Analyzer; GS500-ROX served as the size standard (Applied Biosystems). T-RFLP profiles were interpreted as previously described [13,21].

Statistical analyses. Differences among sites for the studied variables were tested using one-way analysis of variance (ANOVA). A cluster analysis based on the T-RFLP patterns was carried using the software Primer [7] to assess the similarity in community composition of the metal-reducing assemblages in the samples. The cluster analysis defines groups of samples based on the similarity of selected variables (in this case *Geobacteraceae* community composition) and produces a dendrogram that shows how the sites are grouped together.

Results

Physicochemical variables. The results of the physicochemical analyses carried out on the groundwater samples are shown in Table 1. All samples, with the exception of those from well 60, contained relatively high Mn and Fe concentrations. Iron and manganese were highest in the samples from well 82. In all wells, with the exception of number 60, Mn concentrations exceeded 50 µg/l, which in the USA (US EPA 1998) and in the European Community (Directive of the European Council 98/83/EC) is the threshold level for humans. Only in one case (well 82) the iron concentrations were above the legislative limits (200 µg/l in the EC and 300 µg/l in the USA). Iron and manganese concentrations were significantly and positively related ($r = 0.87$, $P < 0.01$).

Total prokaryotes abundance. The abundance of total prokaryotes ranged from 4.89 ± 0.69 to $16.30 \pm 2.42 \times 10^5$ cells/ml in water samples (wells 96 and 97, respectively) and from 0.38 ± 0.23 to $3.96 \pm 1.52 \times 10^8$ cells/g in sediment samples (wells 104 and 97, respectively). Water samples from wells 97 and 104 displayed the highest prokaryotic abundance (ANOVA, $P < 0.01$). In sediment samples, the highest abundance was measured in the sample from well 97 (ANOVA, $P < 0.01$).

Bacterial diversity (number of ribotypes). T-RFLP analyses indicated a high diversity of *Geobacteraceae*, both in water and in sediment. *Geobacteraceae* richness (expressed

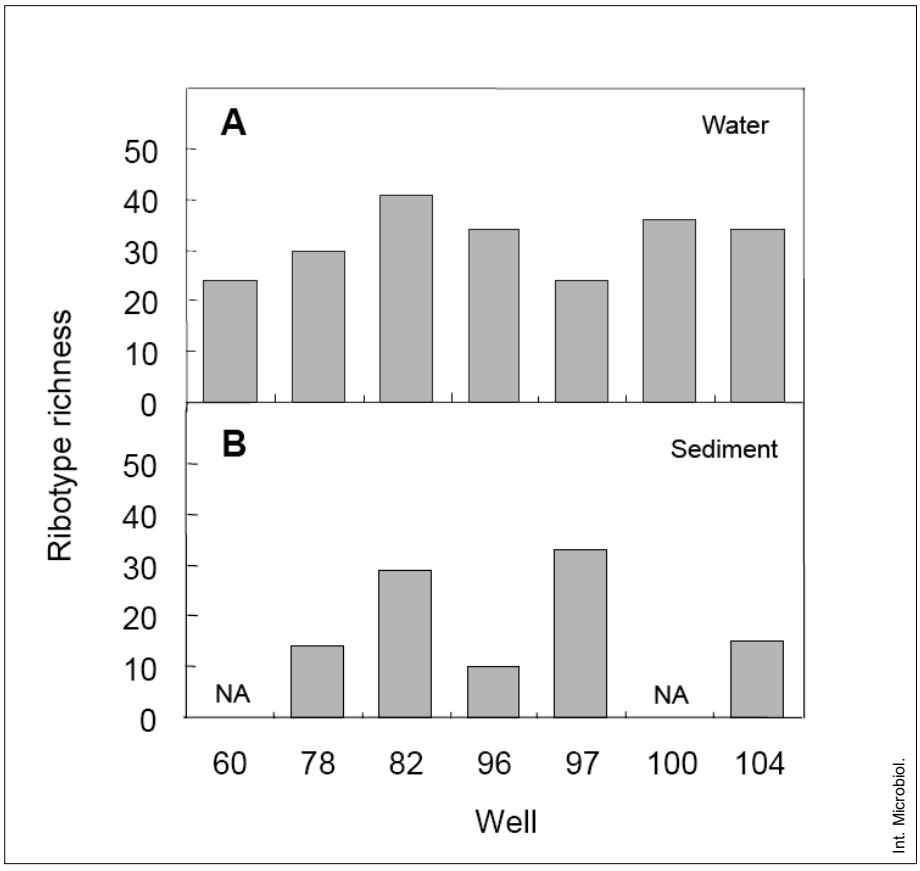

Fig. 2. Phylogenetic diversity (expressed as number of ribotypes) of *Geobacteraceae* assemblages in samples from (**A**) water and (**B**) sediment, as determined by T-RFLP analyses with the *Geobacteraceae*-specific primers. NA, not available.

as total number of ribotypes) ranged from 24 to 41 ribotypes in groundwater samples and from 14 to 33 ribotypes in sediment samples (Fig. 2A,B). In groundwater samples, the diversity of *Geobacteraceae* was highest in well 82, which, as noted above, had the highest iron and manganese content, and lower in wells 60 and 97, which, conversely, had the lowest iron and manganese contents. In the sediments, the highest ribotype richness was observed in the sample from well 97, and the lowest in the sample from well 96.

The analysis of *Geobacteraceae* community composition in water showed that, in the sample from well 60, the 88-bp ribotype accounted for ca. 50% of the total peak height of the electropherogram. Samples from wells 72, 97, and 100 were dominated by the 163-bp ribotype, which accounted for 27, 33, and 22% of the total peak height, respectively. In samples from wells 96 and 104, the 166-bp and 346-bp ribotypes accounted for ca. 40% of the total peak height. The most diversified assemblages were detected in the sample from well 82. These were represented by ribotypes that included those exclusively observed in this sample (e.g., 349 bp; 10% of the total height). Conversely, in the sediments, samples from wells 96 and 104 were dominated by the 75-bp (27–35% of the total peak height) and 347 bp (24–27%) ribotypes. In the sample from well 78, the 164-bp and 258-bp ribotypes accounted for 30 and 28%, respectively. In the sample from well 82, 15, 21, and 20% of the assemblage consisted of ribotypes of 166, 207, and 347 bp, respectively, whereas in the well 97 sample the 348-bp ribotype contributed 37% of the peak height.

Cluster analysis. The cluster analysis revealed that different metal-reducing ribotypes characterized the groundwater samples analyzed (Fig. 3A). Overall, *Geobacteraceae* assemblage profiles displayed a very low similarity among the investigated samples. The unpolluted sample (well 60) displayed <40% similarity with all other samples characterized by a higher iron and manganese content. The remaining samples clustered together, but the similarity level was low. The results also showed that different *Geobacteraceae* assemblages contributed to the investigated sediment sam-

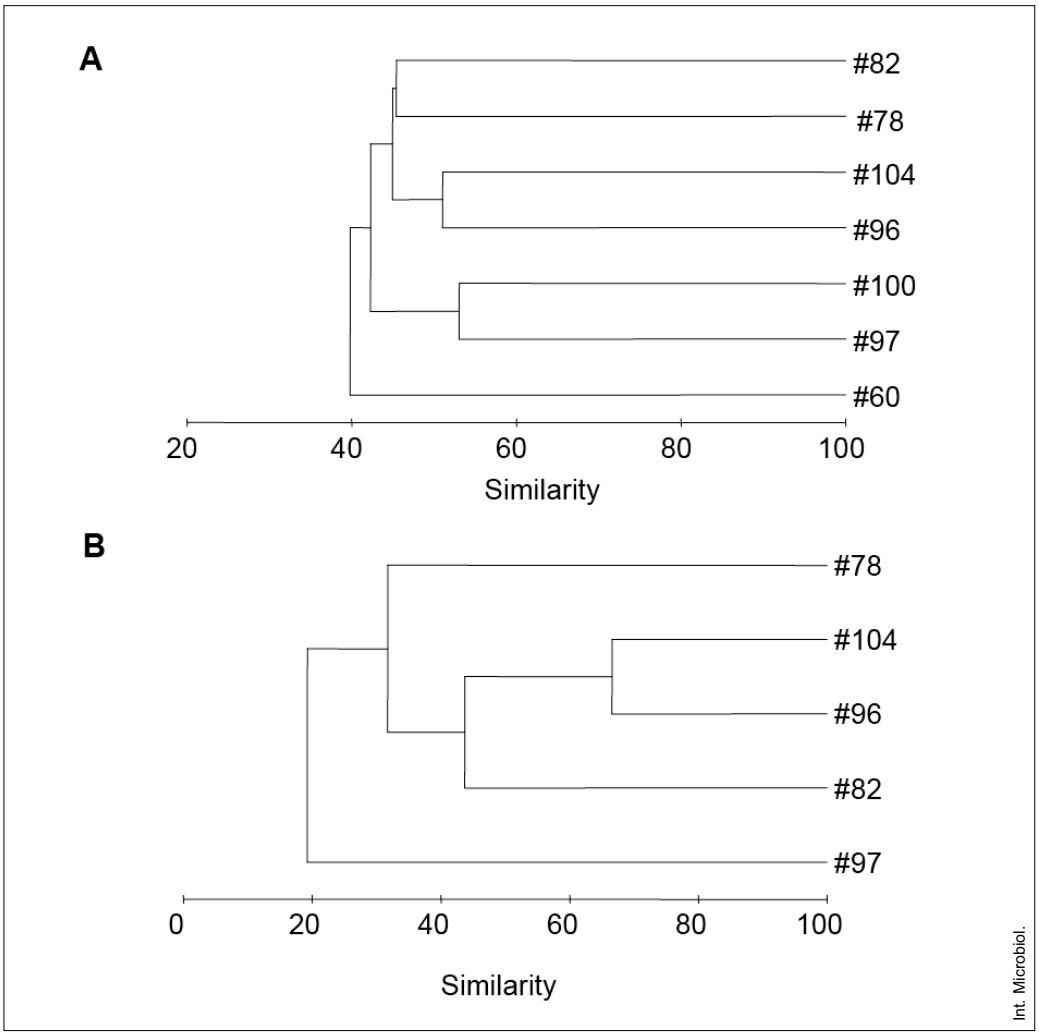

Fig. 3. Cluster analysis of 16S rRNA-based T-RFLP patterns of *Geobacteraceae* assemblages observed in samples from (**A**) water and (**B**) sediment.

ples (Fig. 3B). The samples from well 97 were clearly different (only ca. 30% similarity) from all the other samples. Conversely, in samples clustering together (i.e., those from wells 104 and 96) community composition showed a higher similarity.

Discussion

Iron and manganese concentrations in groundwater depend upon a variety of factors, including metal-reducing processes, that are responsible for the transformation of solid minerals to the dissolved state [12,14,19]. In this study, the diversity of *Geobacteraceae* in groundwater samples characterized by different concentrations of dissolved oxygen and redox compounds was investigated in order to obtain information on the spatial distribution of *Geobacteraceae* and on the relationships between member species and the hydrochemical conditions. Spatial patterns of *Geobacteraceae* diversity were studied using the T-RFLP technique [2,13].

Groundwater samples displayed marked differences in their main physicochemical characteristics. Two samples (from wells 60 and 86) were oxic, while in the others the oxygen levels were below the threshold needed to sustain aerobic microbial processes [26]. Iron and manganese concentrations among the groundwater samples varied by a factor of 10–30. The samples were also extremely diverse in terms of their nitrate and sulfate contents. The high spatial variability in the chemical composition may reflect different input/sources of contamination and/or the result of processes occurring in the groundwater that is in contact with different aquifer materials [14].

Redox processes in aquifers can be investigated through several approaches [4,20,23], all of them offering advantages and limitations [20]. Following the method of MacMahon and Chapelle [26], we identified the potentially dominant redox processes occurring in the studied aquifer. The dominant process in the samples from wells 60 and 96 was likely oxygen reduction, whereas, in samples from wells 78, 97, 100, and 104 denitrification likely predominated. This hypothesis is supported by the very high nitrates concentrations, coupled with the low oxygen level (0.2–0.3 mg/l), i.e., conditions typical for sustaining denitrification reactions [25,26]. Moreover, in these samples, the concentration of Fe was lower than expected for active iron-reducing systems (i.e., >0.1 mg/l [6,26]). Only in the sample from well 82, which contained the lowest nitrate concentration and the highest iron and manganese content, the chemical conditions were indicative of metal-reduction processes.

Metal-reducing *Geobacteraceae* bacterial assemblages were highly diversified. In fact, diversity was higher than previously reported using the same primer set and the DGGE technique to study samples obtained from a petroleum-contaminated aquifer [36]. The higher diversity of metal-reducing bacteria reported here may reflect differences related to the aquifer's characteristics, but also to the higher resolving power of T-RFLP compared to DGGE [15,24,27].

In silico phylogenetic analyses (carried out using the software MiCA [http://mica.ibest.uidaho.edu]) revealed that most of the ribotypes detected by T-RFLP could be putatively ascribed to the genera *Pelobacter*, *Geobacter*, *Desulfuromusa*, and *Desulforomonas*, previously identified as common members of *Geobacteraceae* in subsurface environments [18,37]. Specifically, the 163-bp fragment is common to many *Geobacteraceae* species (belonging to the genera *Desulfuromonas*, *Desulfuromusa*, *Pelobacter*, and *Geobacter*). The 166-bp fragment corresponded to an uncultured deltaproteobacterium (Sva0556). The 207-bp fragment was a *Geobacter* sp., while the 346-bp fragment was assigned to *Desulfuromusa succinoxidans* and to four uncultured deltaproteobacteria. The ribotype of 164 bp was identified as an uncultured *Desulfuromonas* sp. (SB294), while the fragment of 347 bp represented several tens of candidates belonging to the family *Geobacteraceae* (*Pelobacter*, *Geobacter*, *Desulfuromusa* and *Desulforomonas* spp.) and other genera from deltaproteobacteria (e.g., *Desulfofrigus*). The 348-bp fragment was identified as a *Desulfobacteraceae*. All other ribotypes could not be associated to any sequence within the database, suggesting the presence of still-undescribed *Geobacteraceae*.

The higher diversity of metal-reducing bacteria was associated with a higher metal content. Cluster analysis revealed low similarity among groundwater samples, as well as among sediment samples, indicating a considerable variability in *Geobacteraceae* community composition. Samples clustering together were characterized by different redox conditions and contamination levels. For example, in samples from wells 100 and 97, which displayed 55% similarity, oxygen and nitrates contents differed markedly. These results support previous findings highlighting major differences in the community composition of metal-reducing bacterial assemblages also within the same aquifer [17,18,34] and indicate a high turnover diversity of *Geobacteraceae* assemblages in the studied area.

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