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BRIEF REPORT

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Biogeochemical characterization of four depleted gas reservoirs for conversion into underground hydrogen storage

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Abstract

Depleted gas reservoirs are a valuable option for underground hydrogen storage (UHS). However, different classes of microorganisms, which are capable of using free H_2 as a reducing agent for their metabolism, inhabit deep underground formations and can potentially affect the storage. This study integrates metagenomics based on Illumina-NGS sequencing of bacterial and archaeal 16S rRNA and *dsrB* and *mcrA* functional genes to unveil the composition and the variability of indigenous microbial populations of four Italian depleted reservoirs. The obtained *mcrA* sequences allow us to implement the existing taxonomic *database* for *mcrA* gene sequences with newly classified sequences obtained from the Italian gas reservoirs. Moreover, the KEGG and COG predictive functional annotation was used to highlight the metabolic pathways potentially associated with hydrogenotrophic metabolisms. The analyses revealed the specificity of each reservoir microbial community, and taxonomic and functional data highlighted the presence of an enriched number of taxa, whose activity depends on both reservoir hydrochemical composition and nutrient availability, of potential relevance in the context of UHS. This study is the very first to address the profiling of the microbial population and allowed us to perform a preliminary assessment of UHS feasibility in Italy.

INTRODUCTION

In the past two decades, increased knowledge about the effects of greenhouse gases (GHGs) on global warming and climate change pushed for adopting a systematic strategy to reduce both fossil fuel utilization and GHG emissions. The deployment of technologies for energy generation from renewable sources, such as solar and wind power, is one of the answers to climate change. However, the need to balance intermittent power availability and fluctuating energy demand must be satisfied and requires storage solutions targeted at large-scale storage (Götz et al., 2016).

H_2 is currently regarded as a promising green vector for a carbon-neutral economy and the green option is mainly generated through water electrolysis using the surplus of electricity produced by wind and solar energy, with the production of water as the sole byproduct. Green H_2 can be employed in many productive sectors, and different solutions have been proposed for its production, storage, transportation and utilization (Andrews & Shabani, 2012; Nazir et al., 2020; Noussan

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et al., 2020; Panwar et al., 2011). Nowadays, there are several solutions for H_2 storage, including storage in metal tanks above and below ground, in gas networks, in materials (alloys and composites) or deep geological structures (Uliasz-Misiak et al., 2021; Zivar et al., 2021). Underground hydrogen storage (UHS) is considered the most promising option, with salt caverns or depleted hydrocarbon reservoirs used as storage facilities (Benetatos et al., 2021; Jacobs, 2023). However, aquifers, abandoned coal mines, lined hard rock caverns and refrigerated mined caverns are also under consideration (Benetatos et al., 2020; Dopffel et al., 2021; Muhammed et al., 2022). While at present, successful UHS has been limited to salt caverns, storage in depleted hydrocarbon reservoirs remains to be validated under actual operating conditions (Zivar et al., 2021).

Currently, in Italy, all the sites used for natural gas storage are depleted hydrocarbon reservoirs (Eid et al., 2022; Verga, 2018). At present, 15 concessions for storage sites have been issued and four new sites are expected to be put into function; most of them are located in Northern Italy, where gas demand is concentrated (MISE, 2022). The perspective that H_2 may be stored mixed with natural gas, or even in place of natural gas, in the near future, is raising significant interest in the industry but also the need for addressing the issues that are specific to UHS. For instance, the thermodynamic behaviour of H_2 mixtures, H_2 solubility in formation water, the impact of H_2 dispersion when injected underground, and H_2 diffusion are yet to be fully investigated. Additionally, H_2 has a wider range of flammability compared to gases such as CH_4 (Muhammed et al., 2022).

Along with chemo-physical features, the presence of microbial populations able to interact and use H_2 for their metabolisms (i.e., sulphate reduction, hydrogenotrophic methanogenesis, acetogenesis and iron (III) reduction) has been reported as one of the most important factors potentially affecting successful UHS outcomes (Dopffel et al., 2021; Gniese et al., 2014; Ivanova et al., 2007a, 2007b). Among these metabolisms, the reduction of sulphate to hydrogen sulphide (H_2S) (Equation 1), carried out mainly by sulphate-reducing bacteria (SRB), is likely the most harmful because of sulphide reactivity, corrosiveness and toxicity and pore-clogging by precipitate and biofilm formation (Dopffel et al., 2021; Muyzer & Stams, 2008; Panfilov, 2016; Wagner & Ballerstedt, 2013). Moreover, sulphate reduction results in a 100% loss of energy of involved H_2. Similarly, the conversion of H_2 and CO_2 into CH_4 (Equation 2) by hydrogenotrophic methanogens (HM) inhabiting porous media has been frequently reported to cause ~17% loss of energy value due to the conversion of 4 moles of H_2 and 1 mole of CO_2 to 1 mole of CH_4 (Ebigbo et al., 2013; Hagemann et al., 2016; Hattori, 2008; Panfilov, 2010; Toleukhanov et al., 2015; Wagner & Ballerstedt, 2013). Acidification of the reservoir environment and consumption of H_2 are also associated with acetogenesis, carried out by acetogenic bacteria for the conversion of H_2 and CO_2 to acetate (Equation 3; Dopffel et al., 2021; Hattori, 2008; Panfilov, 2016). Conversely, the reduction of ferric iron to its ferrous form (Equation 4), by iron-reducing bacteria (IRB), was reported in relation to corrosion and biofilm formation events (Alabbas & Mishra, 2013; Panfilov, 2016).

$$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O \quad -151.9\,\Delta G^{o'}(\mathrm{kJ/mol}) \tag{1}$$

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \quad -135.6\,\Delta G^{o'}\,(\mathrm{kJ/mol}) \tag{2}$$

$$4\,H_2 + 2\,CO_2 \rightarrow CH_3COOH + 3\,H_2O \quad -104.6\,\Delta G^{o'}(\mathrm{kJ/mol}) \tag{3}$$

$$H_2 + 2\,FeOOH + 4\,H^+ \rightarrow 2Fe^{2+} + 4\,H_2O \quad -228.3\,\Delta G^{o'}(\mathrm{kJ/mol}) \tag{4}$$

Altogether microbiological and chemical features can offer a comprehensive picture of reservoir biochemical characteristics prior to UHS operations and allow for the monitoring of possible changes occurring in the site upon gas injection (Dopffel et al., 2021). These two aspects are strictly interconnected, as microbial metabolisms in natural environments are influenced and shaped by the presence of different arrays of nutrients (e.g., carbon sources and metal ions) and by the physico-chemical parameters characterizing the field (e.g., temperature, pressure, pH, salinity), which select organisms capable of sustaining life in such environments. The chemical characterization of natural habitat provides information regarding the presence and availability of nutrients/inhibitors potentially involved in indigenous microbial metabolisms (Bellini et al., 2022; Haddad et al., 2022; Romero-Güiza et al., 2016). Similarly, as explained above, understanding the microbial biodiversity inhabiting the deep underground is fundamental to the success of UHS. Nevertheless, the microbiology of terrestrial deep subsurface and, in particular, of UGS remains quite an unexplored field. In the last two decades, metagenomic analysis of the 16S rRNA gene and, more recently, the sequencing of functional marker genes proved a valuable approach for the identification of microorganisms present in the subsurface environment, targeting even specific underrepresented microbial consortia (Bomberg et al., 2015; Itävaara et al., 2011; Purkamo et al., 2017; Ranchou-Peyruse et al., 2019). The two main functional marker genes used in this context are key genes for methanogenesis and sulphate reduction,

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that is, *mcrA* and *dsrB*, encoding for the methyl-coenzyme M reductase alpha subunit (mcrA) and dissimilatory sulphite reductase alpha and beta subunits (DsrAB), respectively (Itävaara et al., 2011; Müller et al., 2015; Starnawski et al., 2017).

The present study reports the first, in-depth microbiological characterization of formation waters sampled from four depleted gas reservoirs. To gain a comprehensive picture of the microbial ecology specific to each reservoir and thoroughly characterize the composition and variability of the indigenous microbial populations, a hydrochemical characterization and a combined metabarcoding approach, based on optimized next generation sequencing (NGS) amplicon sequencing pipelines of bacterial and archaeal 16S rRNA and *dsrB* and *mcrA* functional genes, were carried out. Recovered *mcrA* gene sequences were used to implement the current *mcrA* database (Yang et al., 2014), with newly classified sequences obtained from the Italian gas reservoirs analysed. The operational taxonomic units (OTUs) assignment was integrated with a predictive functional study based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Genes (COG) annotations, with a particular focus on hydrogenotrophic metabolisms and their potential in relation to the specific physicochemical conditions of each site. By highlighting the most relevant microbial taxa, including those potentially implicated in H_2 metabolism, this work lays the essential groundwork for a feasibility study and demonstrates the importance of a site-specific assessment for the implementation of UHS.

EXPERIMENTAL PROCEDURES

Sampling sites, substrate characteristics and sample collection

Microbial profiling was carried out on formation waters sampled from four Italian depleted gas reservoirs, named R1–R4, three of which are located in the north of the Country and one is located in the centre. All the geological formations, found at an average depth of 1200–1500 m below ground level, consist of sandstone with good porosity (typically, between 20% and 25%) and permeability (a few hundred millidarcies, corresponding to some 10^{-13} m^2). The original formation pressure was approximately hydrostatic (thus 120–150 bar) and the temperature was in the range of 45–48°C for all the reservoirs. Pressure after depletion due to natural gas production reached 50–70 bars. Sampling involved the collection of some 50 litres of formation water directly in the well at the reservoir level with the BAILER system (provided by Dajan S.r.l.), for each of the four reservoirs under examination. The BAILER system allows for the sampling of groundwater from bottom wells with the presence of formation water at the end of the withdrawal phase. It is a simple instrument, consisting of a tube that is slowly lowered into the water column through a string allowing water to fill it through dedicated drill holes located at the top of the tool. Sample release devices located at the bottom of the tool string (specifically manufactured for this activity) are provided to ease the collection of samples without spillage. To ensure the collection of uncontaminated formation fluids, the following operational precautions were taken during site sampling. All equipment used for fluid recovery and collection was disinfected using a 10X sodium hypochlorite solution prior to the start of the sampling activity. Before the tool string descended to the reservoir level, sterile nitrogen was flushed into the lubricator to ensure the absence of oxygen and avoid the introduction of contaminants, preserving microbial activity for later analysis (not part of this manuscript). After the pull-out of the hole (POOH) of the tool string, pressure relief of the lubricator was adjusted with gradual opening, ensuring a very slow discharge flow to avoid bubbling in the lubricator. The collected fluids were transferred into tanks, previously flushed with sodium hypochlorite and sterile water, and insufflated with sterile N_2 to maintain anaerobic conditions and avoid the introduction of contaminants, and thus preserve the anaerobic microorganisms. After collection, the samples were immediately shipped to the laboratory and stored at 4°C in a dark chamber. Fluid samples taken during the first descent were excluded from laboratory analyses. An aliquot of the formation water was immediately collected for hydrochemical analysis while the remainder was filtered within 24 h after sampling for subsequent microbiological characterization, as described below.

Hydrochemical analysis of formation waters

As soon as the tanks were delivered to the laboratory, part of the water samples were aliquoted into PE bottles and stored at 4°C until shipped to E.L.A. s.r.l. (Asti, Italy) for hydrochemical analysis.

Although the formation water samples were collected at the same depth in the same well, the composition of fluids from at least three tanks (i.e., three descents) was analysed individually to verify the similarity in chemical composition among the different descents. The following parameters were determined: pH and conductivity, main dissolved elements (NH_4^+, PO_4^{3-}, Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}, HCO_3^-, Fe^{2+}, NO_3^-, Br^-, Li^+, Sr, Mn, Ni), carbon content (total carbon, total organic carbon and total inorganic carbon). The carbon content was determined after filtration on paper to eliminate background particle interference. The methods utilized are reported in Table 1.

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TABLE 1 Results of hydrochemical analyses conducted on formation waters derived from the four reservoirs investigated.

Description	R1		R2		R3		R4		Analytical method
	AVG	SD	AVG	SD	AVG	SD	AVG	SD	
pH	7.27	0.17	5.95	0.03	7.72	0.21	5.48	0.04	-
Conductivity (μS/cm)	49,367	896	79,735	3649	49,240	184	123,525	130	APAT CNR IRSA 2030 Man 29 2003
Ammonia nitrogen (NH_3-N) (mg/L)	44.0	1.6	68.5	1.2	52.3	1.7	104.3	2.5	UNI 11669:2017
Calcium (mg/L)	420	22	5	1	557	12	3794	634	UNI EN ISO 11885:2009
Magnesium (mg/L)	257	19	947	97	331	7	1771	306	
Sodium (mg/L)	8533	634	16,898	2183	9860	257	18,472	3029	
Potassium (mg/L)	89	7	519	87	244	4	314	43	
Lithium (mg/L)	2.437	0.175	2.517	0.409	<0.005	0.000	3.383	0.186	
Manganese (mg/L)	0.270	0.059	3.540	0.521	0.587	0.092	2.583	0.508	
Nickel (mg/L)	0.016	0.005	0.035	0.007	0.010	0.001	0.089	0.022	
Strontium (mg/L)	49.22	3.79	163.96	16.75	<0.02	0.00	389.75	16.41	
Phosphate PO_4 (mg/L)	<8	0	<8	0	<8	0	<8	0	APAT CNR IRSA 4020 Man 29 2003
Chlorides (mg/L)	18,711	643	37,055	4551	19,890	513	59,969	775	
Sulphates (mg/L)	<50	0	<50	0	<50	0	126	5	
Bicarbonates (mg/L)	490.56	29.20	772.80	82.30	133.84	6.19	5.67	1.09	
Iron (mg/L)	<5	0.000	145.667	17.172	0.010	0.001	-	0.000	
Nitrates (mg/L)	<50	0	<50	0	<50	0	<20	0	
Bromides (mg/L)	135	5	241	4	109	2	284	4	
Total organic carbon (mg/L)	33.33	4.11	2509.67	40.19	32.13	1.39	229.50	28.39	UNI EN 1484:1999
Total inorganic carbon (mg/L)	198.33	21.82	80.00	14.97	22.52	4.95	30.30	12.49	
Total carbon (mg/L)	231.67	17.93	2589.67	33.77	54.65	4.26	259.75	27.20	

Note: For each reservoir, the average concentration of dissolved elements obtained from the analysis of at least three descents, is reported, together with corresponding analytical method utilized.

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Experimental design

Microbial profiling of R1, R2 and R3 was performed on three formation water samples, obtained from different descents, while microbial characterization of R4 was carried out on a single descent in triplicate. High-throughput sequencing of 16S rRNA bacterial and archaeal genes, together with *mcrA* and *dsrB* marker genes, characterizing methanogenic archaea and SRB, respectively, was carried out.

Moreover, for 16S rRNA gene sequencing a lab control sample aiming at evaluating possible laboratory, environment and/or extraction kit contamination, potentially affecting low biomass samples, was included.

All the samples were analysed in triplicates. The complete list of samples included in this study is provided in Tables S1 and S2.

Processing of samples for metagenomic and quantitative analyses

Immediately after their arrival, the formation fluids were processed by filtration, and the filters were stored at −80°C until the nucleic acids were extracted. To collect microbial biomass, 0.5–1 L groundwater was filtered by vacuum suction using Millipore Express PLUS PES filtering membranes (0.22 μm; Merck Millipore, Burlington, MA, USA). The formation water volume used for each filter was determined based on the water solid content and the outcome of preliminary DNA extraction tests, to select the optimal extraction volumes. The procedure was carried out under a Class II biological safety cabinet, to avoid possible external contamination.

DNA isolation and sequencing

Formation water filters from the four reservoirs and control samples were prepared using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The manufacture extraction protocol was modified by varying the procedure for mechanical cell disruption, performed using the MM 400 Mixer Mill (Retsch GmbH, Haan, Germany) and by lengthening incubation and precipitation times during sample lysis and purification.

The quality and quantity of the DNA extracted were determined using a Genova Nano micro-volume spectrophotometer (Jenway, Cole Parmer Inc) and Qubit fluorimeter (Life Technologies, Carlsbad, CA, USA).

NGS sequencing of the V3-V4 hypervariable region of bacterial and archaeal 16S rRNA genes and *dsrB* and *mcrA* amplicons was carried out by IGA Technology Services (Udine, Italy).

Sequencing of the amplicon pools was performed on an Illumina MiSeq platform using a 300-bp paired-end module. Libraries were prepared by following Illumina 16S Metagenomic Sequencing Library Preparation protocol in two amplification steps: an initial Polymerase Chain Reaction (PCR) amplification using locus-specific PCR primers and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). The list of locus-specific primers utilized in this study is reported in Table S3. Similarly, the preparation of *dsrB* and *mcrA* amplicons (350 and 330 bp, respectively) was optimized by using 0.5 μM of each primer and 10 μL of template in 50 μL reaction volume. PCR protocol consisted of a 3-min initial denaturation at 95°C followed by 35 cycles of 20 s at 98°C, 15 s at 60°C, 15 s at 72°C and a 1-min final elongation. The specificity of signals obtained was confirmed through the Sanger sequencing of amplicons produced from the DNA of *M. maripaludis* and *D. vulgaris* pure cultures.

Upon sequencing, reads were de-multiplexed based on the Illumina indexing system. Where amplicon length was permissive with the respective sequencing length, 3′-ends of pairs were overlapped to generate consensus pseudo-reads, and only overlapping reads were retained. Successively, a clipping routine was applied to remove low-quality bases at 3′ tails. Reads were further retained if they maintained a minimum length of 200 bp. Any primer sequence at 5′-ends was removed and not accounted for during the process.

Metagenomic analysis

Microbiome analysis was performed by IGA Technology Services according to the QIIME pipelines (Caporaso et al., 2010), using the USEARCH algorithm (version 8.1.1756, 32-bit). OTUs are built de novo with a clustering threshold set at 97%, with sequences that passed a pre-filter step for a minimum identity of 90% with any sequence present in the reference database. OTUs are generated with a minimum of two sequenced fragments.

The RDP classifier and Reference database (16S modified GreenGene database, version 2013_8) are used to assign taxonomy with a minimum confidence threshold of 0.50 (DeSantis et al., 2006; Wang et al., 2007).

Regarding *dsrB* and *mcrA* amplicon analyses, taxonomy was assigned using DsrAB—dissimilatory sulphite reductase and *mcrA* gene databases (Müller et al., 2015; Yang et al., 2014). Nevertheless, for *mcrA*, due to the scarcity of annotated sequences in the reference database, upon alignment to the database, the minimum identity filter was removed, to improve read retention and consequent OTU clustering.

Moreover, for both *dsrB* and *mcrA* sequencing results, the average most abundant OTUs (≥1000

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reads) obtained for each reservoir were further aligned against the NCBI database using BLASTN (Zhang et al., 2000) and the taxonomic level was assigned according to the identity thresholds reported by Yarza et al., 2014. Final taxonomy was assigned selecting the most specific common taxonomic level obtained between the two alignments.

Heat maps representing the relative abundance of most abundant OTUs were drawn using the Multiexperiment viewer software (MeV; Howe et al., 2010).

Rarefaction curves end-points and normalization of counts for diversity analysis are set to 50% of the target sequencing coverage (i.e., for 100,000 fragments a cut-off of 50,000 fragments is applied). Samples not satisfying the count threshold were not included in standard alpha- and beta-diversity estimators. Nevertheless, the total count was retained for taxonomic abundance estimation and used accordingly for ad-hoc statistical testing of taxonomic abundance. Alpha diversity was estimated using the Shannon diversity index, whereas beta diversity was estimated with the Bray–Curtis dissimilarity statistic index, and the distant matrix obtained was graphically represented by Principal Component Analysis (PCoA) and displayed by the Emperor tool (https://biocore.github.io/emperor/).

Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Genes (COG) functional categories

Based on previous studies (Bassani et al., 2017; De Francisci et al., 2015), an ad hoc predictive metagenomic analysis was developed to determine KEGG and COG functional category composition of the most abundant genera (≥1%) assigned, having a potential impact on UHS (Galperin et al., 2021; Kanehisa & Goto, 2000). The analysis was carried out by IGA Technology Services (Udine, Italy) as described in Supporting Information.

For both KEGG and COG analyses, the average number of genes assigned to each KEGG and COG category, over the number of genomes considered, was used to compare the predicted functional features of the genera considered in the present study.

Quantification of sulphate-reducing bacteria (SRB), methanogenic archaea and acetogenic bacteria by quantitative PCR (qPCR)

To measure the concentration of SRB, methanogenic archaea and acetogenic bacteria, quantitative PCR (qPCR) analyses were carried out on the same DNA samples used for metagenomic analyses and isolated from R1, R2, R3 and R4 formation water. The analysis targeted the genomic sequences of *dsrB*, *mcrA* and *fhs* amplicons, using the same couples of primers used for *dsrB* and *mcrA* amplicon sequencing, together with primers specific for the *fhs* gene (250 bp) coding for the formyltetrahydrofolate synthetase, a highly conserved enzyme in acetyl-CoA pathway of acetogenesis (Xu et al., 2009). The primer sequences are reported in Table S3. The analysis consisted of a SYBR Green qPCR assay using the QuantiTect PCR Kit (Qiagen, Hilden, Germany), 0.3 μM of each primer and 2.5 μL of template in 25 μL reaction volume. The assays were carried out with the Qiagen Rotor-Q thermal cycler (Qiagen, Hilden, Germany) detecting fluorescence within the range of green (510 nm). qPCR protocol consisted of a 15-min initial denaturation at 95°C followed by 45 cycles of 40 s at 95°C, 40 s at 55°C, 40 s at 72°C and 6-min final elongation. qPCR specificity was tested through melting curve analysis consisting of an initial denaturation for 10 s at 95°C followed by re-annealing at 55°C with a gradual temperature increase up to 95°C while fluorescence was continuously detected. The lowest amplicon concentrations detectable by the used methodology were 1.33×10^2, 1.85×10^2 and 1.76×10^2 copies/mL for *mcrA*, *dsr*, *B* and *fhs*, respectively, so that samples with concentrations below these thresholds are reported as 'not determined (n.d.)'.

RESULTS AND DISCUSSION

Ecological analysis of the reservoirs: Hydrochemical and metagenomic characterization

To gain a comprehensive understanding of the ecology of the reservoirs under investigation, hydrochemical and metagenomic analyses were performed on formation waters (at least three samples were collected for each of the four reservoirs).

It is known that the composition of the formation waters in terms of availability of dissolved nutrients and especially salinity and pH are key factors in determining the composition and diversity of the reservoir-inhabiting microbial population, exerting selective pressure that shapes both microbial life and activity (Gniese et al., 2014). The hydrochemical analysis determined the content of all the main dissolved elements and the carbon content (organic C, inorganic C and total C). Conductivity and pH measurements were also performed. Hydrochemical analysis results showed significant differences in the four formation waters, whose chemical characteristics are summarized in Table 1.

Although within specific ranges acidic pH conditions affect the dissolution and solubilisation of metal elements including Fe, Co, Ni, Cu, Na, Mg, K and Ca in soils and sediments, pH values close to neutrality tend

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to decrease metal solubility (Renpu, 2011; Sharpley, 1991; Tack et al., 1996). With R2 and R4 displaying a more acidic pH (5.5 < pH < 6) when compared to R1 and R3 (pH ~7.7) a higher content of dissolved ions could be envisaged in R2 and R4 formation waters. Consistently, considerable differences in the amount of dissolved mineral ions and salinity were also observed. In particular, R2 and R4 exhibit higher levels of both Na^+ (~8 g/L) and Cl^{2-} (37 g/L and 60 g/L for R2 and R4, respectively), and thus a higher estimated salinity (>40 g/L NaCl) when compared to R1 and R3 (<10 g/L Na^+, ~20 g/L Cl^{2-} and ≤25 g/L estimated NaCl). Although it has been reported that high salinity levels reduce the abundance and diversity of acetogenic and sulphate-reducing microorganisms due to osmotic stress limiting their metabolism, it is necessary to consider that the growth and activity of halophiles belonging to these metabolic groups may not be affected (Bagaria et al., 2013; Jaafar et al., 2009; Muhammed et al., 2022).

R2 and R4 also display higher levels of Mg^{2+} (1 g/L and >1.7 g/L for R2 and R4, respectively) in comparison to the divalent ion concentrations found in R1 (0.25 g/L) and R3 (0.33 g/L). Ca^{2+} levels are measured at ~3.8 g/L in R4, whilst not overcoming 0.55 g/L in R1 and R3. Notably, the content of Ca^{2+} in the R2 reservoir is only 0.005 g/L. As for salinity, concentrations of both Mg^{2+} and Ca^{2+} affect microbial activity. In particular, high levels of the two ions, such as those measured in R2 and R4, are reported to possibly inhibit the activity of methanogenic archaea (Romero-Güiza et al., 2016). Among the chemical species present in formation waters, the ammonia nitrogen (NH_3-N) and the phosphate (PO_4^{-3}) ions are fundamental nutrients for all microorganisms, being the main building blocks for proteins, nucleic acids and energy generation (Gerardi, 2003). Whilst NH_3-N levels are detected in a range between 0.044 (in reservoir R1) and 0.1 g/L (in reservoir R4), PO_4^{-3} concentrations are reported consistently at <0.008 g/L in all four reservoirs. Particularly for what concerns PO_4^{3-}, low levels of this ion may negatively affect the growth and proliferation of the microbial communities. Moreover, sulphate (SO_4^{2-}) content does not exceed 0.05 g/L in all the reservoirs except for R4, where it reaches 0.125 g/L. Similarly, iron levels are negligible in all the formation fluids except for R2 (0.146 g/L). Conversely, none of the reservoirs displays nitrates levels >0.05 g/L.

Considering the carbon sources, different studies highlighted how in different geological formations the coexistence of both heterotrophic and autotrophic microorganisms could occur (Basso et al., 2009; Chen et al., 2017; Dopffel et al., 2021; Flynn et al., 2013; Kimura et al., 2009; Wu et al., 2016), suggesting indigenous reservoir microorganisms can metabolize both organic and inorganic carbon.

Concerning carbon content, R2 formation waters are characterized by remarkable organic carbon and bicarbonate contents (2.5 and 0.773 g/L, respectively) compared to the other reservoirs. The organic carbon sets at 0.23 g/L in R4 and 0.033 g/L in R1 and R3, and the bicarbonate levels vary from 0.006 g/L of R4 to 0.49 g/L of R1. Moreover, the prevalence of organic carbon over the inorganic fractions is noticeable in all the reservoirs apart from R1.

Along with the chemical analysis, metagenomic analyses based on the sequencing of V3–V4 regions of 16S rRNA bacterial and archaeal genes were also performed. The 16S amplicon sequencing has been widely applied for the characterization of deep-subsurface environmental samples (Bomberg et al., 2015; Itävaara et al., 2016; Purkamo et al., 2017). Compared to total metagenomic analysis, it allows for the characterization of samples even starting from a very low concentration of DNA, the required sequencing depth is lower, the bioinformatics analysis is simpler, and the analysis is generally faster and cheaper. However, the technology also has limitations as the use of degenerate primers to amplify hypervariable ribosomal regions can lead to bias as the primers bind to regions that are not 100% conserved in all microorganisms and, in most cases, taxonomic identification is not reliable below the genus level due to the high similarity among the 16S rRNA gene of closely related species. Furthermore, the technique is not quantitative in an absolute sense, and the most represented species may cover the signal of those less present or where the primers bind with less specificity (Cottier et al., 2018; Ghosh et al., 2019). To try to compensate for these limitations and to avoid underestimating or losing archaeal microbial diversity, along with the bacterial V3-V4 region, a target specific for the same archaeal region (Gantner et al., 2011; Takai & Horikoshi, 2000) was chosen in the present study.

For 16S rRNA amplicon sequencing, DNA extraction protocols were adapted and optimized for both rock and formation water samples. Nevertheless, DNA isolation and sequencing from reservoir core sections did not result in reliable microbial profiles (data not shown). This is possibly attributable to the clayey nature of the reservoir rock, affecting the recovery of genetic material and the low concentration of biomass available. For these reasons and based on the previous literature studies limiting microbial profiling to liquid substrates (Bomberg et al., 2015; Itävaara et al., 2011; Purkamo et al., 2017; Ranchou-Peyruse et al., 2019), we decided to focus our analyses on formation water.

Nucleic acids were extracted from formation waters collected from three different descents and considered biological replicates. Library preparation and sequencing were performed in technical triplicates. Detailed sample IDs are listed in Table S4 together with a sum-

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mary of the sequencing results. Overall, 5,889,112 and 7,045,490 reads were produced for the bacterial and the archaeal targets, respectively, with 78% and 82% of them being assigned to OTUs. Both rarefaction curves and Shannon alpha diversity indexes (Figure S1A,B) evidence site-specific microbial populations characterizing the four reservoirs, with R1 displaying the highest diversity, based on bacterial target sequencing. Moreover, beta diversity analysis shows a high statistical correlation between technical and biological replicates, with samples from the same reservoir clustering together (Figure 1A,B). From the comparison of sample profiles, a major consistency within the samples derived from the same reservoir can be noticed, except for R3 where the profile of descent N°4 slightly differs from those of the other descents (Figure 2A and Dataset S1). In agreement with the beta diversity analysis, this consistency is particularly noticeable within R2 samples, where the profiles of all the replicates appear almost overlapping. As expected, a distinct microbial profile characterizes the laboratory control samples, which cluster separately from the reservoir samples (Figures 1A and 2A). The absence or negligible presence in formation water profiles of taxa detected in the laboratory control samples indicates that the 'background noise' due to exogenous contamination known to be present in the extraction kits (Glassing et al., 2016; Salter et al., 2014) did not cover the indigenous microbial population, confirming the reliability of the profiles obtained.

Taxonomic assignments (Figure 2A) generally show a greater similarity between the profiles of R1 and R2 reservoirs, both dominated by the *Synergistales* and *Thermotogales* orders (over 25% relative abundance each), with the majority of the sequences assigned to the *Anaerobaculum* and *Petrotoga* genera, respectively.

Instead, R3 is dominated by the *Clostridiales* order, reaching up to 58% in two (descents N°7 and N°8) of the three samples analysed. Notably, a remarkable fraction of reads obtained with the bacterial-specific universal primers are affiliated with archaeal members of the *Methanobacteriales* order. This finding suggests a predominance of methanogenic archaea in the microbial consortium characterizing the R3 reservoir, although the metabarcoding-based analysis is not quantitative. This hypothesis is supported by the archaea-specific primer (Figure 2B), which only for the

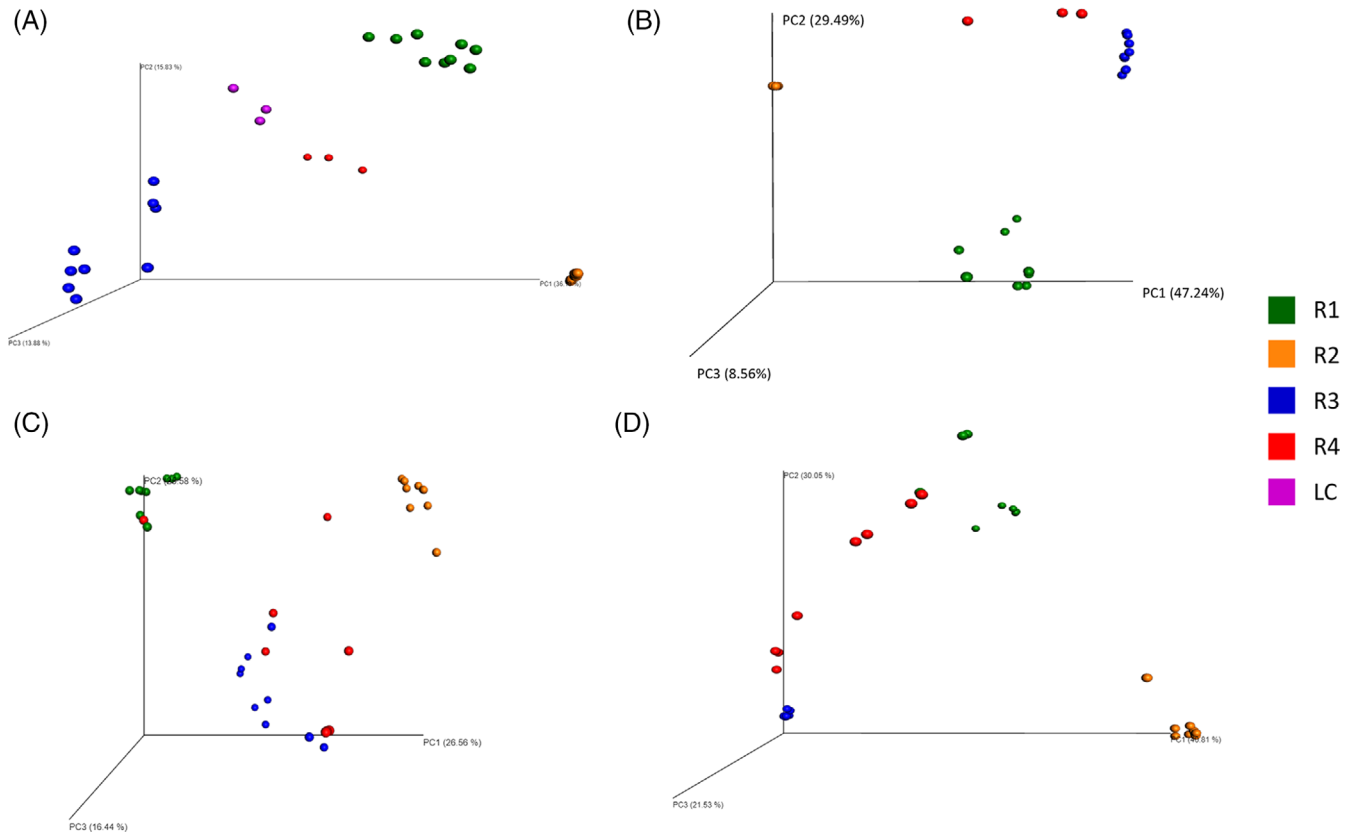

FIGURE 1 Beta diversity PCoA plots generated from 16S rRNA bacterial (A) and archaeal (B) gene, and *dsrB* (C) and *mcrA* (D) amplicon sequencing analyses. The legend of the colour code corresponding to each reservoir under investigation (R1–R4) and lab control samples (LC) is reported on the right part of the figure. Regarding the 16S rRNA archaeal target (B), due to the limited number of reads assigned to lab control replicates, these samples were not included in statistical analyses. For the same reason, R4_1-2_c was not included in statistical analyses of *dsrB* and *mcrA* amplicons sequencing (C and D).

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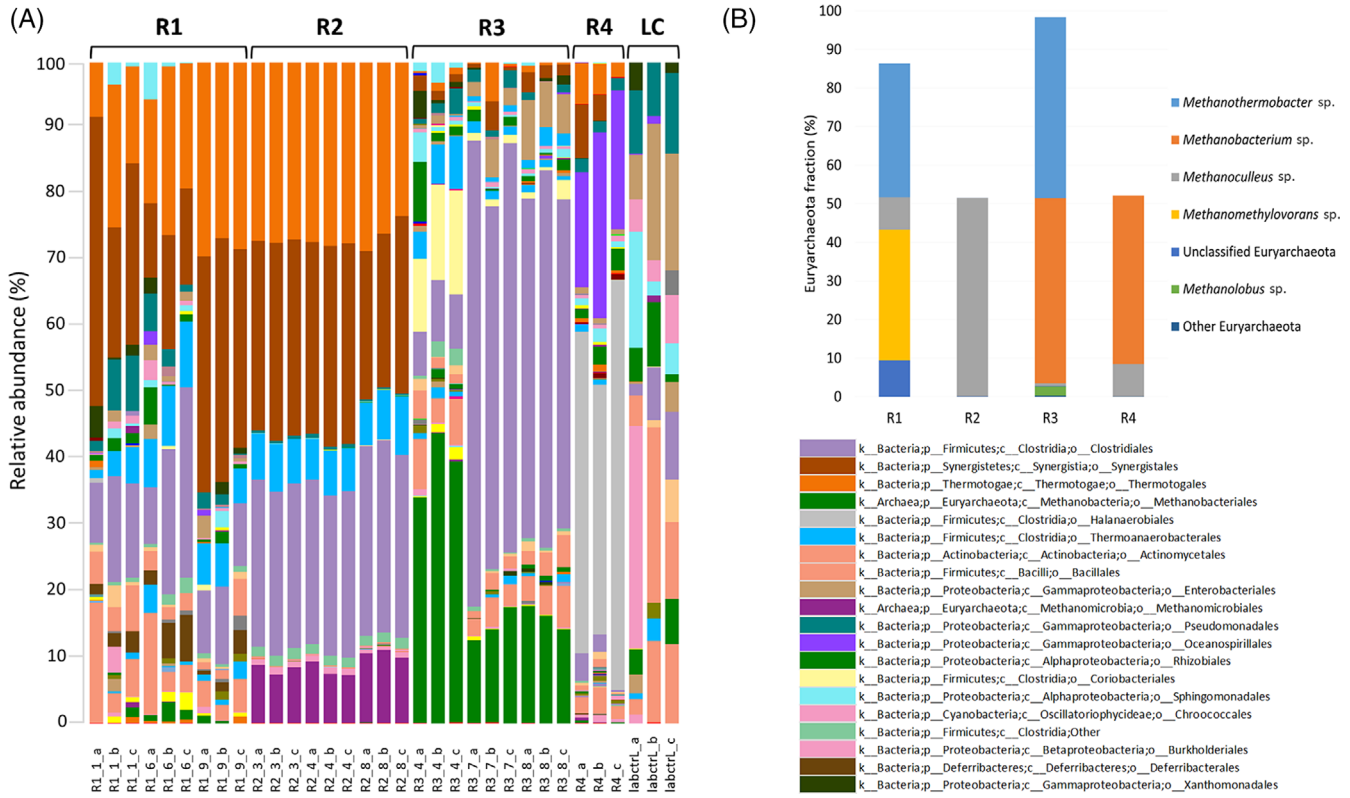

FIGURE 2 (A) Overview of reservoir microbial communities (R1–R4) at the order level, obtained from 16S rRNA bacterial gene sequencing on formation water samples, together with lab control sample (LC) microbial profiles. Sample IDs, indicating source reservoir, descent number and replicate reference are reported at the bottom of the panel. For readability, only taxa occurring at average relative abundance ≥1% are shown in the legend on the right of the panel. (B) Overview of reservoir *Euryarchaeota* population and corresponding reservoir average relative abundance at the genus level, obtained from 16S rRNA archaeal gene sequencing on formation water samples.

R3 reservoir amplifies up to 98% of sequences assigned to the *Euryarcheaota* phylum. This demonstrates the importance of combining the results of two different gene targets to compensate for and compare the results obtained, which are subject to the limitations of the amplicon sequencing technique.

Eventually, the R4 microbial community is mainly characterized by two taxa, that is, the *Halanaerobiales* order (*Firmicutes* phylum, 49% relative abundance), further identified as similar to *Halanaerobium* genus, and the *Oceanospirillales* order (*Gammaproteobacteria* class, 22%).

Overall, the presence of taxa such as *Synergistales*, *Thermotogales*, *Clostridiales* and *Methanobacteriales* in underground formations including aquifers, salt caverns and oil reservoirs has been previously reported (Kimura et al., 2009; Molíková et al., 2022). Nevertheless, despite the ubiquitous presence of such groups of microorganisms, a considerable specificity of each reservoir microbiome composition can be observed. This is particularly noticeable in R4, whose microbial community is dominated by halophilic microorganisms (Dobson & Franzmann, 1996; Liang et al., 2016; Wang et al., 2011). This result is in line with the salinity levels estimated in this reservoir (>40 g/L of NaCl). Salinity levels similar to those of R4 were recorded also in R2. Moreover, in agreement with previous studies, the higher content of carbon sources (2.5 g/L organic carbon and 0.773 g/L bicarbonate) available in R2 could explain the occurrence of homoacetogenic and fermentative bacteria in this reservoir (Hattori, 2008; Maune & Tanner, 2012; Miranda-Tello et al., 2004; Molíková et al., 2022; Thaysen et al., 2021).

Notably, the use of archaeal-specific primers (Figure 2B and Dataset S2) allowed for the detection of archaeal taxa not detected or underestimated with the bacterial target, such as the *Methanomicrobiales* and the *Methanobacteriales* orders. Interestingly, the overall *Euryarchaeota* population, which represents nearly the totality of the archaeal community, was found at different relative abundance among the four reservoirs, ranging from 52% in R2 and R4 to 98% in R3 and displaying mostly distinct profiles for each reservoir (Figure 2B and Dataset S2). Overall, the archaeal population is characterized by low diversity, with the OTUs assigned to only three orders of methanogens, namely *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*. In more detail, the majority of the OTUs were assigned in R1 to *Methanothermobacter*, *Methanomethylovorans* and *Methanoculleus* genera, in R2

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exclusively to *Methanoculleus*, in R3 to *Methanothermobacter*, *Methanobacterium* and *Methanolobus* genera, while in R4 taxa similar to *Methanothermobacter* and *Methanoculleus* were detected.

Alongside the 16S rRNA gene, amplicon-sequencing analyses of *dsrB* and *mcrA* functional genes, specifically targeting SRB and methanogenic archaea, were performed allowing for the profiling and the relative quantification of these groups of microorganisms within the formation fluids collected from each reservoir.

SRB and methanogenic archaea taxonomy were assigned upon alignment to DsrAB—dissimilatory sulphite reductase and *mcrA* gene databases, respectively (Müller et al., 2015; Yang et al., 2014) and ad hoc optimized metagenomic analysis pipelines. Moreover, an additional alignment of the most abundant OTUs against the NCBI database was performed. This analysis led to the implementation of the currently available *mcrA* database (Yang et al., 2014) with newly recovered and classified *mcrA* sequences.

Detailed sample IDs are listed in Table S5 together with a summary of the sequencing results. For each of the targets, 5,712,670 and 6,157,100 reads were sequenced, 88% and 83% of them, respectively, being assigned to OTUs. Overall, 1930 and 1177 OTUs were identified for the *dsrB* and *mcrA* targets, respectively, and the SRB and methanogenic archaea profiles depicted by *dsrB* and *mcrA* amplicon sequencing strongly correlate with those provided by the 16S rRNA gene.

According to the *dsrB* and *mcrA* rarefaction curves based on Shannon alpha diversity indexes, R1 and R2 are characterized by higher microbial diversity compared to the other reservoirs. R4 exhibits the lowest diversity for both targets (Figure S1C,D). Beta diversity analyses show a high statistical correlation between technical and biological replicates belonging to the same reservoir. Slightly higher variability can be observed within samples belonging to the R4 reservoir (Figure 1C,D).

Overviews of both SRB and methanogenic populations of the four reservoirs under investigation are provided in Figure 3 and Datasets S3 and S4.

The *dsrB* gene encodes for the DsrAB enzyme, which is conserved in all SRB and catalyses the last step of the dissimilatory sulphate reduction pathway. The DsrAB phylogenetic tree has four main branches referring to the three major DsrAB protein families, that is, the reductive bacterial type, the oxidative bacterial type and the reductive archaeal type. The fourth branch is so far only represented by the second *dsrAB* copy of *Moorella thermoacetica* (Müller et al., 2015). The analysis of the most abundant OTUs (>1000 assigned reads) here identified revealed that the totality of the taxa detected falls into the reductive bacterial-type DsrAB family (Figure 3A). This family is mainly constituted of bacteria able to utilize sulphate, sulphite or organosulphonates as terminal electron acceptors (Müller et al., 2015). The reductive bacterial-type DsrAB family is further divided into superclusters. In this study, all the highly abundant OTUs classified at least at this

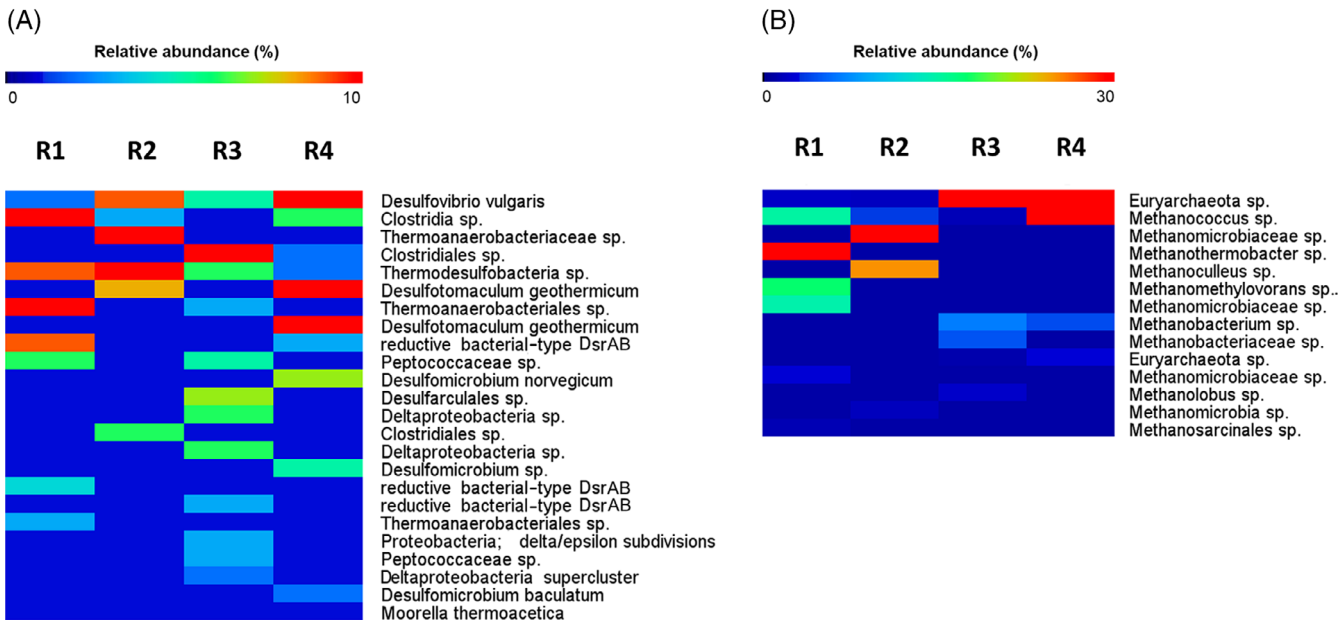

FIGURE 3 Heat maps of reservoir average relative abundance of the most abundant (>1000 assigned reads) SRB and methanogens populating the four reservoirs, obtained from *dsrB* (A) and *mcrA* (B) amplicon sequencing. Correspondence between colours and relative abundance is reported in the scale at the top of each panel.

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taxonomic level were assigned to *Deltaproteobacteria* supercluster, which contains the majority of known bacterial type *dsrAB* sequences, including *dsrAB* sequences belonging to all *Deltaproteobacteria*, *Thermodesulfobacteria* and some members of *Firmicutes* phylum (e.g., *Desulfotomaculum* and *M. thermoacetica dsrAB* copy 1; Müller et al., 2015).

More in detail, the R1 SRB community is dominated by an OTU assigned to the *Clostridia* class and accounting for 42% of relative abundance, followed by a member of the *Thermoanaerobacteriales* order and a member of the *Thermodesulfobacteria* phylum. The latter OTU was also detected at higher relative abundance in R2, together with the *Thermoanaerobacteriaceae* family.

Notably, the R3 SRB community is dominated by only one taxon, assigned to the order *Clostridiales* and covering 43% of the population in terms of relative abundance.

Instead, the majority of the R4 SRB population is constituted by an OTU identified as similar to *Desulfovibrio vulgaris* (44%) followed by two OTUs assigned as similar to *Desulfotomaculum geothermicum* (13% each).

In agreement with the 16S rRNA gene sequencing, the SRB community populating all the reservoirs, apart from R4, is dominated by members of the *Clostridia* class, over the *Deltaproteobacteria* class. These taxa were also recently found in oil fields, sulphate and CH_4-rich groundwater from deep boreholes, and marine sediments tested for H_2S production (Bomberg et al., 2015; Briones-Gallardo et al., 2022; Rizzo et al., 2020; Varjani & Gnansounou, 2017).

As expected, the *mcrA* amplicon sequencing results mostly correlate with those of 16S rRNA archaeal gene sequencing, pointing out the comparable occurrence of *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* orders, thus confirming the reliability of the obtained results. Furthermore, the specificity of the *mcrA* target allowed for the detection of taxa not detected by the 16S rRNA target, such as the *Methanococcus* genus and other OTUs assigned at a less deep classification level (Figure 3B).

Specifically, in R1 an OTU assigned to the *Methanothermobacter* genus covers the majority of the community (38% of relative abundance), followed by *Methanomethylovorans* and *Methanococcus* genera, and by an unclassified member of the *Methanomicrobiaceae* family. The other reservoirs are dominated by only one or two OTUs, in accordance with the low diversity already found with the ribosomal target. However, the *mcrA* sequencing allowed us to identify in R4 an OTU classified as similar to the *Methanococcus* genus, previously undetected.

Notably, the average most abundant OTU detected among all the microbiomes was classified only at the phylum level as a member of the *Euryarchaeota* phylum. While the alignment against the available *mcrA* database (Yang et al., 2014) assigned this OTU to the *Methanomicrobia* class, the subsequent BLAST analysis pointed out a similarity to the *Methanothermobacter* genus, belonging to the *Methanobacteria* class. Because of this discordance, a deeper taxonomic level could not be assigned.

This demonstrates the importance of implementing the available databases so that the sequences found can be assigned to more specific taxonomic levels.

Microbial processes of interest for the storage of H_2 in four Italian depleted gas reservoirs

H_2 is a key energy source for deep-surface microbial processes, especially in environments with limited alternative electron donors. For this reason, a deep understanding of the microbiology of the H_2 cycle is dramatically important for subsurface engineered environments, where H_2-utilizing microorganisms might significantly affect gas storage and pipeline facilities (Dopffel et al., 2021). Previous studies identified sulphate reduction, methanogenesis, acetogenesis and iron reduction as the four main microbial metabolisms possibly taking place within a storage site (Gniese et al., 2014; Hagemann et al., 2016; Panfilov, 2016).

Metagenomic analyses conducted on formation waters collected from four Italian depleted gas reservoirs unveiled the occurrence of microbial taxa capable of performing these microbial processes in each site under investigation. Moreover, qPCR analyses detected the presence of up to 10^3 and 10^4 copies/mL of methanogenic archaea (*mcrA* functional gene) and acetogenic bacteria (*fhs* functional gene) in two and three of the four reservoirs analysed, respectively (Table 2).

However, as discussed below, the presence of these microorganisms does not necessarily reflect a risk of metabolism activation. Indeed, metagenomic analyses carried out on genomic DNA do not provide information on actual microbial activity, which would be provided by metatranscriptomic studies and activity assays. Moreover, for a comprehensive evaluation of the potential impact of these microorganisms on UHS, the reservoir hydrochemical characteristics and nutrient availability must be taken into account. The presence of nutrients or organic compounds at concentrations not sufficient to support long-term microbial growth, or conversely growth-inhibiting (e.g., high salinity), could reduce the potential risk of microbial activation.

Table S6 summarizes the main microbial taxa found in the four reservoirs object of this study and the biological processes in which they could be potentially involved thus posing an issue for UHS operations. Moreover, Figure 4 provides a summarized graphical representation of KEGG and COG functional assignment of metagenomic data. The complete overview of

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TABLE 2 Results of quantitative PCR (qPCR) analyses conducted on the genomic DNA isolated from the formation waters derived from the four reservoirs investigated.

	dsrB (copies/mL)		*mcrA* (copies/mL)		*fhs* (copies/mL)	
Reservoir	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
R1	n.d.	-	2.42E + 03	1.32E + 03	1.09E + 04	5.69E + 02
R2	n.d.	-	n.d.	-	1.79E + 02	1.25E + 02
R3	n.d.	-	5.72E + 02	4.21E + 01	1.04E + 04	5.94E + 02
R4	n.d.	-	n.d.	-	n.d.	-

Note: For each reservoir, the average concentration of each amplicon is reported. The lowest amplicon concentrations detectable by the used methodology were 1.33×10^2, 1.85×10^2 and 1.76×10^2 copies/mL for *mcrA*, *dsrB* and *fhs*, respectively, so that samples with concentrations below these thresholds are reported as 'not determined (n.d.)'.

the results obtained with KEGG and COG analyses is provided in Dataset S5.

Reduction of sulphur compounds

Reduction of sulphate and sulphur compounds with consequent H_2S production (Equation 1) by sulphate-reducing microorganisms is associated with 100% loss of energy of involved H_2, corrosion of technical equipment, acidification of reservoir formation fluids and serious health risks for operators (Dopffel et al., 2021). Not only H_2, but also organic compounds can be used as electron donors for the conversion of sulphur compounds to H_2S, enlarging the variety of microorganisms posing a potential risk for storage operations beyond hydrogenotrophic species (Gniese et al., 2014).

By looking at the microorganisms identified within the formation water collected from the four sites, several taxa were found possibly associated with the reduction of sulphur compounds. Among them, *Petrotoga* (*Thermotogales order*) and *Anaerobaculum* (*Synergistetales* order) represent the most abundant genera, detected in all the reservoirs. Interestingly, these taxa were previously isolated from fluids produced from oil reservoirs, being able to grow at moderate halophilic conditions (Maune & Tanner, 2012; Miranda-Tello et al., 2004; Pournia et al., 2018). This explains their occurrence at the remarkable salt levels detected in the reservoirs analysed. These microorganisms can ferment glucose and other organic compounds to acetate, CO_2 and H_2, being able to reduce sulphur compounds to H_2S (Lien et al., 1998; Maune & Tanner, 2012; Miranda-Tello et al., 2004; Pournia et al., 2018). Similarly, almost half of the OTUs characterizing the microbial community of R4 were assigned to the halophilic *Halanaerobium* genus. This genus can grow at >150 g/L NaCl concentrations and has been previously detected in hydraulically fractured oil and gas wells; it can degrade guar gum (a polysaccharide used in fracture fluids) and other carbohydrates and to produce acetate and sulphide (Liang et al., 2016; Lipus et al., 2017).

Consistently, the predictive analysis of KEGG and COG functional categories assigned to these taxa revealed the presence of an enriched number of genes for the transport and metabolism of carbohydrates and amino acids.

Moreover, this analysis highlighted a high number of genes involved in the sulphur metabolism pathway in the previously mentioned most abundant taxa (e.g., *Halanaerobium* and *Petrotoga*), and also in less abundant taxa, described in the literature as capable of reducing sulphur compounds to H_2S (Table S6, Figure 4A and Dataset S5). Among them, the *Desulfotomaculum* (*Clostridiales* order) genus, to which OTUs were assigned limitedly to the microbial population of R3, possesses the complete set of genes for the reduction of sulphate to H_2S (module ID: M00596).

Moreover, the higher specificity of amplicon sequencing based on the *dsrB* functional gene allowed for the detection of additional taxa not detected through 16S rRNA gene sequencing. For example, an OTU assigned to the *Thermodesulfobacteria* phylum was detected in all the reservoirs, and a member of the *Desulfarculales* order was found only in R3. These SRBs require the presence of H_2 or organic compounds as electron donors to reduce sulphate or other sulphur compounds to H_2S, for example, some *Desulfarculales* species can grow autotrophically with H_2/CO_2. Moreover, these taxa are known to tolerate moderate salinity levels (An & Picardal, 2014; Davidova et al., 2016; Hamilton-Brehm et al., 2013; Jeanthon et al., 2002; Sonne-Hansen & Ahring, 1999; Sun et al., 2010), possibly explaining their presence at the salinity levels characterizing the Italian reservoirs analysed.

Additionally, several taxa such as *D. vulgaris* and *Desulfomicrobium* species were found limited to R4, and, according to KEGG functional analysis, they display the whole set of genes for the reduction of sulphate to H_2S. The occurrence of these microorganisms had been previously reported in marine sediments and deep aquifers used for natural gas storage (Briones-Gallardo et al., 2022; Ranchou-Peyruse et al., 2019).

Conversely, qPCR analyses pointed out the occurrence of SRB carrying the *dsrB* gene at concentrations

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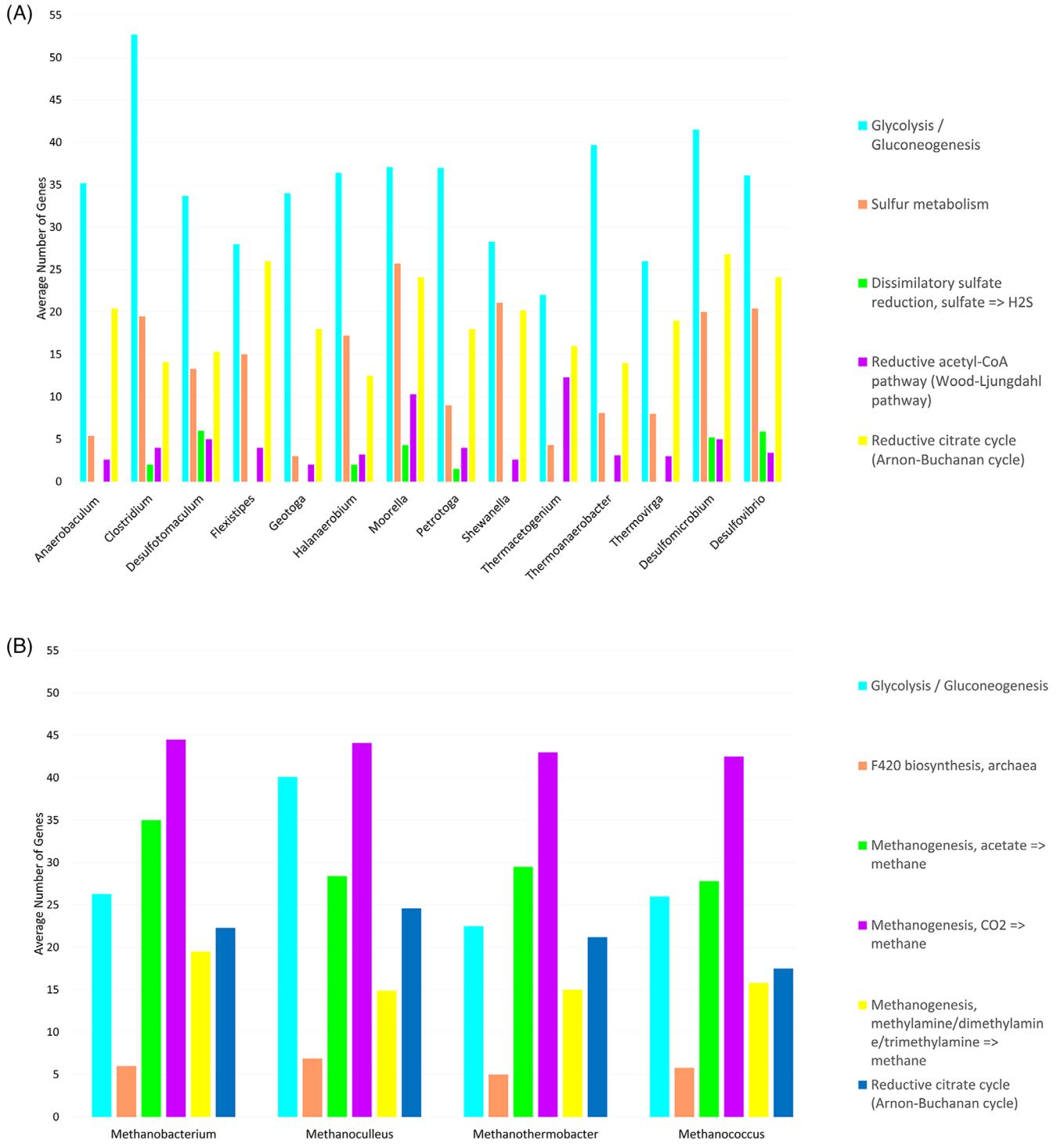

FIGURE 4 Graphical representation of KEGG predictive metagenomic analysis results performed on most abundant (≥1% relative abundance, calculated from biological and technical replicates of the same reservoir) bacterial (A) and archaeal (B) genera detected through 16S rRNA and functional marker gene sequencing, and having a potential impact on UHS. The *y*-axis refers to the average number of genes assigned to each KEGG pathway or module reported in the legend, over the number of genomes considered. Only a selection of the most relevant categories in the context of UHS is displayed in the figure, whereas the complete overview of KEGG and COG analysis results is provided in Dataset S5. Specifically, categories related to sulphur metabolism and sulphate reduction could be possibly associated with H_2 consumption, H_2S production and consequent detrimental effects on storage operation and facilities. Similarly, the representation of modules related to methanogenesis from different substrates reflects the potential of HM for H_2 consumption through hydrogenotrophic methanogenesis. The occurrence of genes involved in the WLP could be putatively associated with the activity of homoacetogenic and SAO bacteria, potentially resulting in H_2 consumption and reservoir acidification and syntrophic interaction with HM, respectively. Eventually, the illustration of categories related to carbohydrate metabolism and reductive citrate cycle points out metabolic features (e.g., fermentation of sugar compounds) and a potential advantage in sub-optimal conditions. The putative impact at the reservoir level of each represented taxa is described in detail in Table S6.

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below the detection limit, suggesting an actual low concentration of these microorganisms in all the reservoirs analysed (Table 2).

Hydrogenotrophic methanogenesis

Loss of H_2 and energy value (about 17%–18%) can be potentially caused by the activation of the methanation process carried out by hydrogenotrophic methanogenic archaea, which can oxidize 4 moles of H_2 and reduce 1 mole of CO_2 to produce 1 mole of CH_4 (Equation 2; Bellini et al., 2022; Ebigbo et al., 2013; Gniese et al., 2014; Hagemann et al., 2016; Panfilov, 2010; Toleukhanov et al., 2015; Wagner & Ballerstedt, 2013).

Regarding the presence of HM within the reservoir archaeal community, few taxa were detected at high relative abundance. Specifically, methanogenic archaea belonging to the *Methanobacteriales* order, and further identified as similar to *Methanothermobacter* and *Methanobacterium* genera, cover nearly the totality of the R3 archaeal community, and almost half of R1 and R4 populations. Moreover, OTUs assigned to the *Methanomicrobiales* orders, and more specifically the *Methanoculleus* genus, were detected in all the reservoirs, covering more than half of the R2 archaeal community. These microorganisms are capable of producing CH_4 exclusively from the reduction of CO_2 with H_2 or formate (Boone, 2015; Lai, 2019), while some *Methanoculleus* species are capable of using secondary alcohols as electron donors. Consistently, the analysis of KEGG categories pointed out the presence of a higher number of genes associated with hydrogenotrophic methanogenesis (~44 genes; module ID: M00567), compared to those associated with the other methanogenic routes (up to 35 and 20 genes for acetoclastic and methylotrophic pathways, respectively; Figure 4B and Dataset S5), thus confirming the prevalence of this methanogenic metabolism in the taxa under analysis. As expected, the predictive functional analysis of archaeal taxa shows the presence of the whole pathway for cofactor F420 biosynthesis (module ID: M00378), which catalyses crucial redox reactions in methanogenesis (Grinter & Greening, 2021). Moreover, in agreement with previous studies (Anderson et al., 2009; De Francisci et al., 2015), the *Methanoculleus* genus is characterized by a higher number of genes for the Glycolysis / Gluconeogenesis pathway and the Reductive citrate cycle (Arnon–Buchanan cycle), one of the major carbon-fixing pathways in anaerobic microorganisms, compared to the other HM detected (40 and 25 genes, compared to average 25 and 20 genes). This could potentially provide this genus with an advantage when growing in sub-optimal conditions, such as those of R2 (estimated salinity levels >40 g/L of NaCl and pH values <6), and thus possibly explain its dominancy within the archaeal population of this reservoir.

Conversely, the other most abundant methanogens detected, belonging to the *Methanosarcinales* order (*Methanomethylovorans* and *Methanolobus* genera), are not considered a direct issue in the context of UHS, as they are reported to utilize methyl compounds for CH_4 production, not being able of using H_2 and CO_2 or acetate (Jiang et al., 2005; Lai, 2019).

Moreover, based on amplicon sequencing targeting the functional gene *mcrA*, an OTU assigned to the *Methanococcus* genus, which accounts for more than half of the R4 archaeal community, was detected in all the reservoirs. The results of the analysis of the KEGG modules associated with methanogenesis in *Methanococcus* are perfectly in line with those reported for the other HM detected, depicting a prevalence of genes for hydrogenotrophic methanogenesis. Species of this genus produce CH_4 using exclusively H_2 or formate as electron donors (Whitman, 2015).

In agreement with 16S rRNA archaeal gene sequencing pointing out a higher *Euryarchaeota* relative abundance in R1 and R3, compared to the other two reservoirs, qPCR analyses revealed the occurrence of methanogenic archaea at 2.42×10^3 and 5.72×10^2 copies/mL in R1 and R3 respectively, while in R2 and R4 *mcrA* amplicon concentrations were reported below the threshold of detection (Table 2).

Acetogenesis

Acetogenic bacteria utilize 4 moles of H_2 as an electron source for the reduction of 2 moles of CO_2 to acetate (homoacetogenesis; Equation 3; Hattori, 2008), overall leading to slight environment acidification and consumption of stored H_2, or fermenting organic compounds to acetate without implying the consumption of H_2 (acetogenesis; Fu et al., 2019). The drop in pH determined by the activity of acid-producing microbes, homoacetogens or heterotrophs, may result in the dissolution of carbonate and other minerals and, therefore, in the alteration of rock porosity and increase of dissolved CO_2, which can be used by microorganisms as carbon source (Dopffel et al., 2021).

In the present study, homoacetogenic bacteria belonging to the *Clostridiales* order, such as highly abundant members of the *Clostridiaceae* family, were detected in all the reservoirs. Moreover, within the *Thermoanaerobacterales* order, unclassified members of the *Thermoanaerobacteraceae* family and *Thermacetogenium* and *Moorella* genera were found, even though at a low relative abundance and limited to some of the reservoirs considered. As shown by the KEGG and COG analyses, these microorganisms present the complete or nearly complete set of genes for

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the Wood–Ljungdahl pathway (WLP; module ID: M00377); in fact, they include homoacetogenic bacteria producing acetate from CO_2 and H_2. In the presence of a H_2-consuming microorganism, such as HM, utilizing the products of the reaction, some members of these taxa are also able to use the WLP in the opposite direction through syntrophic acetate oxidization (SAO) of acetate to CO_2 and H_2, possibly establishing syntrophic relations with HM (Hattori, 2008; Manzoor et al., 2018).

Moreover, acetogenic bacteria able to ferment organic compounds to acetate and other fermentation products, such as previously mentioned *Petrotoga*, *Anaerobaculum* and *Halanaerobium* genera can be potentially involved in reservoir environment acidification.

Consistently, according to qPCR analyses, acetogenic bacteria accounted for 1.09×10^4, 1.79×10^2 and 1.04×10^4 copies/mL in R1, R2 and R3 respectively, while they were undetectable in R4 (Table 2).

Iron reduction

Eventually, the reduction of insoluble ferric iron to soluble ferrous iron, by IRB using H_2 (Equation 4) or organic compounds as electron donors can have serious consequences on storage facilities. The reduction of insoluble ferric iron may enhance biocorrosion phenomena, through the removal of the protective corrosion scales formed on an exposed surface and through biofilm formation (Alabbas & Mishra, 2013; Weber et al., 2006). Moreover, the reaction of ferrous iron with SRB-produced H_2S can lead to insoluble FeS-precipitates and therefore a reduction of the permeability of porous rocks and pore-clogging (Dopffel et al., 2021; Gniese et al., 2014).

In the present study, only one taxon able to perform iron-reducing metabolism was identified. Specifically, the *Shewanella* genus (*Alteromonadales* order of *Gammaproteobacteria* class), was found by bacterial 16S rRNA sequencing at a low relative abundance and limitedly to R3. *Shewanella* is capable of performing the so-called dissimilatory ferric iron reduction, that is, the transfer of electrons to ferric iron reducing it to ferrous iron without assimilating it. It can secrete soluble electron shuttles to reduce ferric oxide without coming into direct physical contact (Lemaire et al., 2020; Weber et al., 2006).

Critical discussion of reservoir microbial ecology with respect to biochemical issues related to UHS

The integration of data from the hydrochemical analyses (i.e., dissolved nutrient availability), metagenomic analyses, and KEGG and COG functional category assignment allows us to critically discuss the potential biochemical issues associated with the implementation of UHS at each site under consideration. The synthesis of our results and observations is the following.

Overall, the R1 microbiome exhibits the highest microbial diversity, being dominated by taxa capable of reducing sulphur compounds to H_2S. Nevertheless, because of the low content of sulphate detected in this reservoir, the substrate available for these microorganisms is likely to be quickly consumed, allowing the establishment of other metabolisms (Dopffel et al., 2021; Muyzer & Stams, 2008; Ranchou-Peyruse et al., 2019; Sela-Adler et al., 2017). Bicarbonate levels (500 mg/L) may sustain the activation of acetogenic metabolisms or hydrogenotrophic methanogenesis, even though homoacetogenes may be favoured by their metabolic flexibility to utilize a vast variety of substrates (Thaysen et al., 2021) and by their higher cell number compared to HM, enlightened by qPCR.

Similarly, R2 presents a taxonomic profile mainly associated with acetogenesis, fermentation and reduction of sulphur compounds. Metagenomic results were confirmed by qPCR analyses, pointing out the occurrence of acetogenic bacteria at concentrations higher than that of methanogenic archaea and SRB. Additionally, the pH characterizing this field (average values <6) is known to favour the availability of mineral ions, such as Fe^{2+}/Fe^{3+}, Co^{+}, Ni^{2+}/Ni^{3+}, Cu^{+}/Cu^{2+}, Na^{+}, Mg^{2+}, K^{+} and Ca^{2+}, in the formation water due to their leaching from rock formation (Renpu, 2011; Sharpley, 1991; Tack et al., 1996). These data, together with the high levels of organic carbon and bicarbonates detected in the reservoir, could sustain homoacetogenesis and fermentation of organic compounds to acetate. Eventually, IRB were not detected in R2 despite the presence of higher iron concentrations compared to the other fields.

Overall, the R3 microbial community is dominated by HM, occurring at a relative abundance higher than for any of the other reservoirs analysed, and by acetogenic bacteria. As previously presented, a remarkable fraction of reads sequenced with the bacterial target was assigned to methanogenic archaea. Nevertheless, the low concentration of carbon in the formation water, highlighted by hydrochemical analyses, may limit the activation of hydrogenotrophic and acetogenic metabolism to the initial phases of the storage (Sela-Adler et al., 2017). Notably, IRB were found only in this reservoir even though at low relative abundance (~1%); additionally, the negligible content of soluble iron present in R3 formation waters may suggest that the activation of iron reduction metabolism is unlikely. However, mineralogical analyses performed on reservoir rocks, which are currently ongoing, will help identify sources of carbonates and minerals (such as insoluble iron), potentially sustaining metabolic activities at early stages or over time.

Eventually, the R4 microbial profile and qPCR results strictly correlate with the hydrochemical analyses pointing out an estimated salinity of ~47 g/L of

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NaCl and the higher sulphate content (0.125 g/L) when compared to the other reservoirs. The R4 microbial community exhibits a low diversity, being dominated by halophilic bacteria not detected in the other fields, with few identified taxa capable of reducing sulphur compounds to H_2S. In addition, the presence of HM was also identified. Similar to R3, the low carbon content detected in the formation water may sustain the activation of these microbial groups limited to their initial contact with H_2.

It is worth mentioning that, during the last few years, several research projects investigated the potential of UHS. Among them, the Underground Sun Storage project led by the Austrian gas company RAG is the first reported example of the actual injection of H_2/natural gas mixtures in a depleted gas reservoir. Within this project, reservoir microbial community composition and activity, upon exposure to H_2 mixtures, were evaluated at the laboratory scale in high-pressure bioreactors, and at the reservoir level in field tests (Underground Sun Storage Final Report, 2017). Although metatranscriptomic data pointed out the dominancy of methanogenic archaea, followed by SRB, together with CH_4 production and CO_2 consumption, UHS in the tested field was considered feasible, with 82% of the injected H_2 being recovered after 5 months, no H_2S production and no negative impact on existing storage facilities (Underground Sun Storage Final Report, 2017).

These results are quite encouraging because very similar findings were obtained from the comprehensive assessment of metagenomic and physicochemical features carried out in this study on four Italian gas reservoirs, highlighting the occurrence of specific groups of microorganisms of potential relevance in the context of UHS, but also the apparent lack of conditions for long-term thriving of such microorganisms. This approach represents the first indispensable step for the microbial characterization of underground reservoirs for UHS. An appraisal of microbial activity evolution in the presence of H_2 or H_2 mixtures will be performed in reactor assays mimicking reservoir conditions to complement and validate the obtained results, and biogeochemical modelling will be applied to corroborate predictive functional annotations and to estimate the long-term impact of microbial activities.

CONCLUSIONS

Given the paramount importance of the evaluation of the microbiological risks associated with UHS and the expected site-specificity of each reservoir microbial population, this study aimed at filling the information gap on taxonomic composition and potential metabolic dynamics characterizing the deep microbial ecosystems of the Italian depleted gas reservoirs.

The present work represents the first comprehensive characterization of four Italian gas reservoirs in terms of taxonomic characterization and composition (namely, microbial diversity and relative abundance) of indigenous microbial consortia with a focus on the major microbial groups that pose a potential risk from the perspective of UHS, in particular sulphate-reducing bacteria and hydrogenotrophic methanogens. Furthermore, the availability of dissolved elements able to favour these processes was assessed. The evaluation of potential microbiological issues associated with UHS is supported by the predictive functional assignment of metagenomic data based on the KEGG and COG categories.

Although the four geological formations were characterized by comparable thermo-dynamic conditions (i.e., temperature and pressure) and lithologies, metagenomic analyses highlighted the site-specificity of the microbial community of each reservoir under investigation. Two of the fields analysed are dominated by microorganisms capable of undertaking acetogenesis and sulphur compound reduction, the third is mostly populated by hydrogenotrophic methanogens, and the fourth is dominated by halophilic bacteria, able to grow at >150 g/L NaCl levels. These findings reinforce the importance of a targeted microbiological study for each site under investigation for H_2 storage. Despite the occurrence of several taxa of interest in the context of the UHS, the potential impact of these microorganisms on stored H_2 and storage facilities has to be evaluated considering the specific reservoir hydrochemical characteristics (e.g., salinity levels) and nutrient (e.g., carbon sources, and sulphates and iron levels) availability. All the reservoirs analysed have low or negligible sulphate levels, three fields show low carbon contents and two of them have moderated salinity levels (>40 g/L NaCl). Microbial activity assessment and biogeochemical modelling analysis, which are currently in progress and will integrate metagenomic data with metatranscriptomic and quantitative information, will allow us to estimate the potential extent of microbial metabolism activation and H_2 consumption at the reservoir level. Microbial activity experiments at actual reservoir conditions will be targeted to evaluate the effect of different gas mixtures on the microbial communities populating the formation fluids. The biogeochemical modelling will integrate chemical, taxonomic and metabolic activity data to simulate and predict the risk of the activation of the metabolic pathways of interest over time and confirm or adjust the obtained predictive functional annotation. The integration of all these data will allow us to reliably evaluate the microbiological risk associated with UHS for each site under investigation.

Such a comprehensive approach can be applied to the assessment of UHS feasibility of other currently uncharacterized depleted reservoirs.

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AUTHOR CONTRIBUTIONS

Ilaria Bassani: Conceptualization (supporting); data curation (lead); formal analysis (lead); investigation (equal); methodology (equal); validation (lead); visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Ruggero Bellini:** Investigation (equal); validation (supporting); writing – original draft (equal); writing – review and editing (equal). **Arianna Vizzarro:** Investigation (supporting); validation (supporting); writing – review and editing (supporting). **Christian Coti:** Project administration (supporting); supervision (supporting); writing – review and editing (supporting). **Vincenzo Pozzovivo:** Conceptualization (supporting); methodology (supporting); writing – review and editing (supporting). **Donatella Barbieri:** Writing – review and editing (supporting). **Candido Fabrizio Pirri:** Funding acquisition (lead); project administration (lead); supervision (equal); writing – review and editing (supporting). **Francesca Verga:** Funding acquisition (lead); project administration (lead); supervision (supporting); writing – review and editing (lead). **Barbara Menin:** Conceptualization (lead); funding acquisition (equal); investigation (equal); methodology (equal); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

CONFLICT OF INTEREST STATEMENT

We declare that Christian Coti, Vincenzo Pozzovivo and Donatella Barbieri are employed by Snam Stogit S. p.A., a company specializing in underground natural gas storage.

DATA AVAILABILITY STATEMENT

The obtained reads have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB61320 (https://www.ebi.ac.uk/ena/browser/view/PRJEB61320) and ENA BioSample IDs are listed in Tables S4 and S5.

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