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"Innovative high pressure/high temperature, multi-sensing bioreactors system for microbial risk assessment in underground hydrogen storage"

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ABSTRACT

This study addresses the microbial risks associated with Underground Hydrogen Storage (UHS), a critical component in the transition towards renewable energy systems, by employing an innovative multi-reactor system (Bio-xplorer) to simulate UHS conditions in two Italian reservoirs. The microbiological risk assessment (MRA) of Reservoir A and B was evaluated by subjecting them to gas mixtures of 10 % H_2 and 90 % CH_4, and 99 % H_2 and 1 % CO_2, respectively.

In Reservoir A, the stability of pressure and temperature, the negligible optical density, and lack of microbial metabolites suggested a low risk of microbial activation. Molecular analyses confirmed the absence of sulphate-reducing bacteria (SRB) and limited growth of hydrogenotrophic methanogens (HM). Similarly, in Reservoir B, the absence of SRB and limited occurrence of HM indicated a low microbiological risk. Overall, the present work supports the safe and efficient implementation of UHS, a promising mitigation technique for climate change, using an innovative tool for MRA.

1. Introduction

The greenhouse effect is a critical environmental concern, and it is of utmost importance to take effective measures to mitigate its impact. In September 2020, the European Commission proposed ambitious targets for greenhouse gas (GHG) emission reduction and the share of renewable energies by 2030 and set long-term goals for achieving a carbon-neutral economy by 2050 [1,2]. To meet these targets, carbon-neutral electricity production is crucial, and the share of renewable energy in the energy mix needs to increase significantly [3–5]. However, the intermittent nature of renewable sources as solar and wind energy poses challenges for grid stability and necessitates the development of large-scale energy storage and reserve production capacity [6–9]. To address these challenges, Power-To-X (p2X) technologies, including Power-To-Gas (p2G), have been developed to store excess renewable energy in the form of various chemical compounds, such as hydrogen (H_2) [10,10,11,11,12]. In fact, hydrogen production from renewables is a promising and extensively studied approach [13,14]. In Italy, guidelines set by the Ministry for the Economic Development (MISE) aim to increase the share of renewable H_2 in final energy consumption to 20 % by 2030 and 2050 [15]. Additionally, there is an expected increase in natural gas (NG) demand for energy storage purposes [16].

Among various gas storage technologies, underground hydrogen storage (UHS) in oil and natural gas reservoirs, aquifers, and salt caverns has been identified as a cost-effective solution for peak-shaving capacity [17–20]. However, these geological formations, although characterized by extreme conditions, have been found to harbour microbial populations, including hydrogenotrophic methanogens (HM), acetogenic bacteria (AB), and sulphate-reducing bacteria (SRB) [14,21]. These microorganisms have metabolic pathways that could affect the storage operations, particularly with respect to: (i) H_2 consumption through hydrogenotrophic processes and, as a result, loss of the energy value; (ii) damage to plant and technical equipment due to biocorrosion and clogging caused by the production of damaging chemical species,

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biofilm formation and biomass accumulation; (iii) microbial-induced acidification of the underground fluids through the formation of hydrogen sulfide (H_2S) and organic acids produced by sulphate reduction and acetogenesis; (iv) pore-clogging and decrease of permeability of porous rocks caused by FeS-precipitates and microbial biofilm (iii) safety risks to operators mainly due to the production of H_2S, which is highly toxic [14,22,23].

During the last years, an increasing number of studies and applied research projects investigated the biochemical aspect related to UHS in geological formations. Geological formations currently assessed for hydrogen storage are mainly depleted hydrocarbon reservoirs, deep aquifers and artificial salt caverns [14,22]. For example, Dopffel et al. (2023) investigated the microbial activity of a brine derived from a Northern Germany salt cavern, upon 100 % H_2 incubation at 30 °C, in batch tests, in the presence or absence of different carbon sources. Without nutrient supplementation, H_2 loss resulted comparable to those of negative controls (i.e., sterile water), whereas higher H_2 consumption (11 %), together with H_2S production and pH increase (up to 8.5; possibly reducing long-term microbial activity), were recorded in batches supplemented with nutrients. In the absence of external carbon sources H_2S was not detected, even though putative FeS precipitates were observed, the lack of nutrients representing one of the main limiting factors recorded during the study. Additionally, cultures supplemented with 10 % CO_2 and 90 % H_2 did not show CH_4 or acetate production [23].

In Italy, there are currently fifteen UGS facilities located in depleted natural gas reservoirs [24]. Previous studies have characterized the chemical and microbial composition of formation water identifying indigenous microorganisms of interest for UHS. However, information on the activity and growth of these microorganisms is limited and need to be further investigated. A thorough examination of the interactions between injected gases and pre-existing minerals, gases, ions, bacteria, and other factors is essential for ensuring safe storage operations with minimal leakage risk. It is also crucial to maintain the purity of hydrogen and prevent its conversion into other gases [23,25,26].

To address these issues significant fundamental and experimental research is being undertaken at different scales and in different disciplines, from microscale to medium scale in laboratory before the develop of pilot testing on reservoirs [27–29]. Laboratory-scale experiments are typically conducted at low pressures, and batch enrichment cultures are developed to evaluate the activity and growth of HM, SRB, and AB microorganisms present in reservoir microbial populations [30,31]. Moreover, reactor systems are employed to simulate UHS at lab scale. These systems predominantly operate in batch or fed-batch mode, at pressures and temperatures higher than ambient [32–37].

The objective of this study was to develop an innovative multi-reactor system (*Bio-xplorer, H.E.L. London*) to investigate microbial reactions under reservoir conditions, and its functionality was evaluated across two reservoirs.

The reactor was designed to operate in a multi-sensing mode, allowing for the measurement of multiple parameters such as biological measurements on microorganisms, as well as the composition of gases and liquids. The functionality of the system is to replicate the conditions found in underground gas reservoirs by performing a controlled high pressure and temperature (up to 200 bar,g and 150 °C respectively) testing campaign in the bioreactors which contains a multiphase system consisting of solid rock and formation water samples injected with hydrogen mixtures. To achieve this, cylindrical rock samples was obtained through deep coring, together with the formation water, inserted into the reactors, and subjected to the desired conditions of temperature, pressure and hydrogen mixture to reproduce the reservoirs conditions.

Whitin this study, we conducted a comprehensive investigation on the microbiological risks associated with UHS by replicating storage conditions of two Italian reservoirs, namely Reservoir A and Reservoir B. The experimental activities were performed using the Bio-xplorer system capable of maintaining the high operational temperatures and pressures of the two reservoirs of interest. The aim was to evaluate the possible activation of microbial populations present in both rock samples and formation fluids and assess their potential effects on the storage of hydrogen.

Overall, the results obtained from the two testing campaigns provide insights into the microbial populations, gas compositions, volatile fatty acids concentrations (products of microbial metabolism), and hydrochemical characteristics of the tested systems along the experimental campaign. These findings contribute to a better understanding of the microbiological risks associated with UHS and can inform the development of strategies to mitigate potential issues and ensure the safe and efficient storage of hydrogen in underground reservoirs.

2. Material and methods

2.1. Samples for reactors inocula

The reactors were inoculated with liquid (formation water) and solid (core section) samples collected from two Italian depleted gas reservoirs, i.e., Reservoir A and B, both located in the North of the Country. The geological formations, found at an average depth of 1200–1500 m below ground level, consist of sandstone with good porosity (between 20 % and 25 %) and permeability (a few hundred millidarcies, corresponding to some 10^{-13} m^2). The original formation pressure was approximately hydrostatic (i.e., 120–150 bar) and the temperature was in the range of 45–48 °C. Pressure after depletion due to natural gas production reached 50–70 bars.

Formation fluids were collected anaerobically and aseptically from the wells, whereas core sections were sampled and immediately cryopreserved until the start of the trials. Before inoculation, sample handling and preparation were carried out under a steady flow of H_2, CO_2, and N_2 using an anaerobic workstation.

2.2. Reactor operations

The reactor tests were conducted using a customized reactor system consisting of two 1-liter stainless steel vessels (*Bio-xplorer*). These vessels were capable of maintaining high operational temperatures (up to 150 °C) and pressures (up to 200 bar). The system can operate in both batch/fed-batch and continuous modes for both the gas and liquid phases.

2.2.1. Bio-reactors overview

The core of the equipment design are the high-pressure bioreactor (HPB) vessels, which are specially designed to keep the rock samples in place and prevent side flow and contamination. Due to geometrical, pressure, temperature and microbial constraints, these bioreactors required a new customized design. The reactors were manufactured using type 316 stainless steel and have a separated lid. All the system components (vessel, lid, fittings, valves and pipelines) were constructed using materials capable of withstanding potential corrosion phenomena, such as those possibly induced by produced H_2S. The lid contains ports with high-pressure fittings for all gas and liquid feeds, including the inoculum, as well as for pressure monitoring and safety features. The control system allows the user to select either the top and/or the bottom temperature probe reading, or the circulator temperature as input for the temperature control loop. An additional pressure transducer is fitted at the bottom of the reactor to monitor pressure differences and prevent the polymer cuff breaking under pressure.

2.2.2. Frame and general assembly

The reactors are mounted on a wheeled floor-standing stainless steel frame, which also provides support for electronic housing, pumps, Mass Flow Controllers (MFCs), and other devices. In the case of specific safety specifications, the listed devices could be positioned on a separate frame to ensure separation from the reactors. The electronics rack and

circulator are placed outside the fume-hood. A lifting mechanism is incorporated to facilitate the handling of the relatively heavy bioreactors. The configuration includes a fixed head and a movable bioreactor body. The entire bioreactor system, including piping and auxiliary devices, has been designed and tested to operate up to 200 bar,g and 150 °C. Fig. 1 shows the designed and manufactured High-Pressure Bioreactors system and the detailed PID (piping and instrumentation diagram) is reported in Fig. S1 of the Supporting information.

2.2.3. Inoculum preparation and inoculation procedure

Before inoculation (Fig. 2), the inlet/outlet lines and reactor vessels were sanitized by washing them with autoclaved water and 70% ethanol solution. After the sanitation phase, the thermal in-situ sterilization procedure started by gradually increasing the temperature to 121°C for 3h. Then, the vessels were cooled to 0 °C to maintain the rock inoculum frozen during the inoculation procedure.

Core rock samples were cleaned from drilling mud residues [38] and cut into slices in a biological hood to ensure sterile conditions. The rock slices were wrapped in a mesh bag and stored at −80 °C until their insertion into the reactor. The sterilized vessels were flushed with nitrogen (N_2) to create anaerobic conditions. During reactor seeding, the vessels were opened, and the core rocks were placed inside the reactors. The vessels were closed, and anaerobic formation fluids were pumped into the vessels to reach a total working volume of 600–700 ml (rocks + fluid). Continuous N_2 flushing was performed during seeding operations to maintain anaerobic conditions. The reactor temperature was gradually increased to 50 °C while continuously flushing with methane (CH_4). Once the operational temperature was reached, the reactors were pressurized and maintained at high pressure with CH_4 for 2 months to promote microbial revitalization [32].

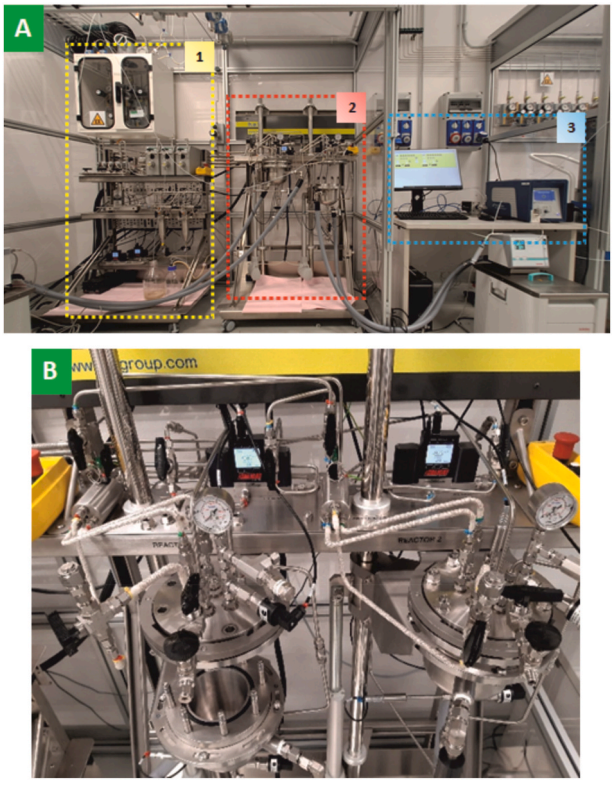

Fig. 1. High-Pressure (HP) and high-temperature (HT) Bio-xplorer configuration: A-photo detail of (1) feeding and downstream area, (2)bioreactors area and (3)monitoring/control and gas analyser area; B-photo detail of bioreactors vessels and lids.



Fig. 2. Rock inoculum preparation and its positioning inside the vessel.

2.3. Testing conditions in high-pressure BioReactor

The customized reactor system consisted of two stainless steel vessels working in parallel (R1 and R2), allowing for two separate experiments to be conducted simultaneously. Two different hydrogen (H_2) mixtures (R1: 10%H_2 - 90%CH_4; R2: 99%H_2 - 1%CO_2) were tested for the reservoirs A and B. The reactors were set to reach and maintain a temperature of 50°C and a pressure of 150 bar,g. After the acclimatization of the inoculum described before, the H_2 mixtures were injected into the systems until the complete shift of the reactors' inner atmosphere. The two way valve used for the gas sampling is also used during this phase for the gas extraction by linking the valves to the vent line. This approach allows for the modification of the headspace atmosphere within the reactor, ensuring that the liquid phase within the saturated rock remains undisturbed. The experiments were conducted under constant temperature and pressure with working gas mixtures for 4 months. The stability of the pressure depends on the possible microbial activity.

Then, a total of 6-month operation was conducted for both R1 and R2. Samples of the headspace gas and liquid phase were collected at specific time points, as reported in Table 1.

Gas samples are collected using a two-way ball valve and a needle valve located on the reactor lid (Fig. 1 and Fig. S11 in SI). This system allows for the extraction of gas samples without disturbing the liquid phase. The assembly consisting of these two valves can be directly connected to the Gas Chromatograph (GC) for sample analysis, or it can alternatively be attached to a syringe system for manual sampling.

Table 1
Bioreactors sampling time for gas and liquid phases.

Sampling time points	Day	Operation	Gas sampling		Liquid sampling	
			R1	R2	R1	R2
t0	1.5	Inoculation – beginning phase 2	x	x	x	x
t1	62	Adaptation phase – end phase 3	x	x		
t2	65	Shift of gas phase – beginning phase 5	x	x		x
t3	130	Incubation – middle phase 5	x	x		x
tf	184	End of experiment – end phase 6	x	x	x	x

The liquid phase is extracted from the bottom of the reactor through an extraction system comprising the Back Pressure Regulator (BPR), a cyclone, and an outlet solenoid valve (Fig. S12 in SI). The BPR is slightly opened, allowing the liquid phase to exit from the reactor's bottom and accumulate in the cyclone. When the liquid level inside the cyclone reaches 50 ml, the BPR closes, and the outlet solenoid valve opens, enabling the sample to be collected.

2.4. Hydro-chemical analysis

Hydro-chemical analyses were carried out by E.L.A. s.r.l. (Asti, Italy) independently on at least three descents of formation fluids. pH, conductivity, dissolved element concentration and carbon content were determined, pointing out a pH lower than 6 for both formation waters, an estimated salinity level of approximately 50 g/L NaCl (ranging from 47 g/L-0.8 M of RA to 54 g/L-0.92 M of RB) and a total carbon content of 0.26 and 2.80 g/L in RA and RB, respectively. Moreover, sulphate was detected only in RA formation water (0.13 g/L), while resulting under the method detection limit in RB. The methods utilized and the complete overview of the results achieved are reported in Table 2.

2.5. Cell optical density

Cell density was measured in 1 ml liquid samples at a wavelength of 600 nm with a Cell density meter Model 40 (Fisher Scientific, New Hampshire, USA). Collected samples of cell density was screened against that of sterilized formation water samples.

2.6. Volatile fatty acids (VFAs) analysis

Volatile fatty acids (VFAs) play an important role in microbial metabolism. Their concentrations were evaluated using high-performance liquid chromatography (HPLC) with a Thermo-Fisher Dionex Ultimate 3000 system (Fisher Scientific, New Hampshire, USA). VFAs were quantified by eluting the sample through an HPLC column (Metab AAC, ISERA GmbH, Düren, Germany) with 9 mM H_2SO_4 at a flow rate of 0.6 mL/min and a temperature of 40 °C adapting the methodology previously described by Ref. [39]. Calibration standards were prepared using individual stock solutions of the VFAs.

Table 2
Results of hydro-chemical analyses conducted on formation waters derived from the Reservoirs A and B, and corresponding analytical methods utilized. For each reservoir, average values and standard deviations were calculated from the results obtained from at least three descents of formation fluids collected and analysed independently.

Description	Reservoir A		Reservoir B		Analytical Method
	Average	St. Dev.	Average	St. Dev.	
pH	5.48	0.04	5.97	0.00	
Conductivity (μS/cm)	123525	130	97976	452	APAT CNR IRSA 2030 Man 29 2003
Ammonia nitrogen (NH3–N) (mg/L)	104	2	98	5	UNI 11669:2017
Calcium (mg/L)	3794	634	8550	250	UNI EN ISO 11885:2009
Magnesium (mg/L)	1771	306	1320	0	
Sodium (mg/L)	18472	3029	21046	582	
Potassium (mg/L)	314	43	383	4	
Lithium (mg/L)	3.38	0.19	5.10	0.15	
Manganese (mg/L)	2.58	0.51	4.58	0.36	
Nickel (mg/L)	0.09	0.02	0.04	0.00	
Strontium (mg/L)	390	16	232	1	
Phosphate PO4 (mg/L)	<8	0	<8	0	APAT CNR IRSA 4020 Man 29 2003
Chlorides (mg/L)	59969	775	45893	2907	
Sulfates (mg/L)	126	5	<50	0	
Bicarbonates (mg/L)	5.67	1.09	395	8	
Iron (mg/L)	–	–	113	8	
Nitrates (mg/L)	<20	0	<50	0	
Bromides (mg/L)	284	4	250	2	
Total Organic Carbon (mg/L)	230	28	2720	40	UNI EN 1484:1999
Total Inorganic Carbon (mg/L)	30	12	75	45	
Total Carbon (mg/L)	260	27	2795	85	

2.7. Gas chromatography analysis

Headspace gas composition in the reactor were measured using gas chromatography with a MicroGC Fusion system (Inficon, Bad Ragaz, Switzerland). Gas samples were collected at different times during the test and stored in gas bags or directly sent to the gas chromatograph. Gas concentrations were determined for H_2, O_2, N_2, CH_4, CO_2, and H_2S.

2.8. qPCR methodologies for the quantification of HM and SRB

Quantitative polymerase chain reaction (qPCR) was used to measure the abundances of hydrogenotrophic methanogens (HM) and sulphate-reducing bacteria (SRB) during the batch cultivation of the formation waters. The qPCR protocol [40] targeting genomic sequences of mcrA and dsrB genes was adopted. The protocol was performed on a Qiagen Rotor Gene-Q and involved a Sybr Green qPCR assay using the Quantitect Sybr Green Kit (Qiagen, Hilden, Germany). The amplification conditions included an initial denaturation step, followed by denaturation, annealing, elongation, and a final elongation step. Melting curve analysis was performed to test the specificity of the amplification. The lowest detectable copy numbers for mcrA and dsrB were determined, and statistical analysis was conducted using a one-way ANOVA test.

3. Results and discussion

The study aimed to investigate the potential impact of microbiological activity on UHS by replicating different storage conditions in the two reservoirs. The difficulty in assessing the extent of microbiological risk in UHS lies in the fact that this risk depends on a large number of different geochemical parameters, such as salinity and availability of organic carbon and other nutrients but also T and pH. Furthermore, it is crucial to consider the very composition and variability of the indigenous microbial community, which is shaped and affected by the aforementioned parameters. To date, there is no universally recognised categorization for identifying the level of microbiological risk, although some recent studies have moved in this direction (Thaysen et al. (2021) and Thaysen et al. (2023), attempting to outline a methodology for categorizing the level of risk based on a collection of microbial growth constraints. These studies have been performed in the framework of the HyStorPor - Hydrogen Storage in Porous Media Project, which aimed at estimating the microbial activity of hydrogenotrophic microorganisms and hydrogen consumption in 47 depleted oil and gas reservoirs located in the East Irish Sea and in the UK and Norwegian North Sea. The assessment has been carried out on the basis of the physicochemical parameters limiting microbial life, such as (e.g., temperature, salinity, and pressure constraints; (Thaysen et al., 2021). This study highlighted that, in 38 of the 47 fields examined, estimated microbial H_2 consumption constituted a negligible to a small percentage of the stored H_2 (<0.01–3.2 %), with five fields being considered sterile, due to their temperature (>122 °C; Thaysen et al., 2021). Similarly, in 2023, the analysis was extended to 75 sites located on the UK continental shelf, categorizing nine of the fields as sterile (temperature >122 °C), 35 and 22 as low (temperature >90 °C) and medium (temperature >55 °C and salinity >1.7 M NaCl) risk, respectively, and only nine as high-risk (temperature <55 °C; Thaysen et al., 2023).

About to the risk categorization based on temperatures reported by Thaysen, our reservoirs could be classified as potentially high risk.

However, when considering other fundamental parameters such as salinity and pH, which are crucial in determining microbial abundance and diversity in the subsurface, our conditions present a slightly acidic pH range. This acidity tends to inhibit the activity of certain microorganisms, such as methanogens (Thaysen et al., 2021). Regarding salinity, the reservoirs in consideration exhibit values slightly lower than 1 M. These high salinity values could hinder other metabolic activities such as homoacetogens and HM (Thaysen et al., 2021). Furthermore, sulphate levels detected in formation fluids (Table 2) resulted below the activation threshold indicated suggested by Thaysen et al., 2021. Thus, it seems improbable unlikely that SRB activity could be sustained over the medium to long term.

Given the paramount importance of the evaluation of the microbiological risk associated with UHS and the expected site-specificity of reservoir physicochemical and microbiological features, this study aims to provide a comprehensive assessment of the microbiological risk related to the storage of H_2 in two Italian reservoirs. Such a comprehensive approach can and should be applied to the assessment of UHS feasibility in other uncharacterized reservoirs.

3.1. R1 testing campaign: reservoir A, 10 % H_2 - 90 % CH_4

The first part of this section is dedicated to report the results obtained from the control and monitoring software during the development of the batch test, such as for example the pressures (p) and temperatures (T) of the gas phase in the headspace and of the liquid phase at the bottom of the reactor. An example of the monitored trends for these parameters (T, p) along the development of the test is reported in Fig. 3.

In Fig. 3 the trends of temperatures and pressures along the different operational phases previously described are reported as follows:

- Phase 1: Sanitization – Sterilization – Inoculation (5 days)
  - 1-1: sterilized water cleaning;
  - 1–2: ethanol solution cleaning and sterilized water washing;
  - 1–3: autoclave;
  - 1–4: anaerobiosis and inoculation;
  - 1–5: final N_2 feeding;
- Phase 2: CH_4 and formation water feeding (12 days);
- Phase 3: maintenance with CH_4 at high pressure (2 months);
- Phase 4: change of gas composition from 100%CH_4 to 10%H_2 - 90% CH_4 (5days);
- Phase 5: maintenance at high pressure with gas mixture (4 months);
- Phase 6: end of test, sampling of gas/liquid/rock (10 days)

Fig. 3 also displays trends of pressures and temperatures in vessel headspace and outlet throughout the whole period of the tests. Throughout the different phases of the experiment it was observed that reactor pressure trends were stable indicating no appreciable changes that could be attributed to microbial activation, with the few small variations observed relatable to the gas-liquid dissolution equilibria and slight temperature/pressure changes which occur during the long experimentation.

Results obtained from analysis of samples collected during the inoculation and at the opening of R1 reactor after 6 months of batch adaptation and incubation are reported in Table 3 and Fig. 4.

By the end of the experiment, parameters related to possible microbial activation were measured. O.D. readings are employed both on pure and mixed microbial cultures in order to provide measurement of cellular density in liquid samples. It was observed that after 6 months incubation, a slight increase of measured O.D. was reported with values ranging between 0.22 and 0.24 in R1 fed with H_2:CH_4 mixtures at 10 %:90%v/v respectively. Although increases in O.D. are usually related to an increase in microbial biomass concentrations, it has to be considered that when handling complex samples characterized by presence of multiple microbial species and high content of sediments, measurement of O.D. might give an understatement of the real occurring

Table 3
Operational conditions and experimental parameters monitored for the liquid phase at the beginning (T_0) and end (T_f) of R1 operations used to evaluate activation of the microbial population.

Sampling time	T_0	T_F
Reactor - H_2:CH_4 Ratio (%/%)	R1 - 10:90	
Operational T(°C)	50	50
Operational P (bar)	150	150
pH	5.58 ± 0.3	7.03 ± 0.3
O.D. (600 nm)	0.00	0.25
VFA_{total}	N.D.	N.D.
mcrA (copies/ml)	Rocks: 5.21 × 10^3	Rocks: N.D.
	Fluid: 4.10 × 10^2	Fluid: N.D.
dsrB (copies/ml)	Rocks: N.D.	Rocks: N.D.
	Fluid: N.D.	Fluid: N.D.

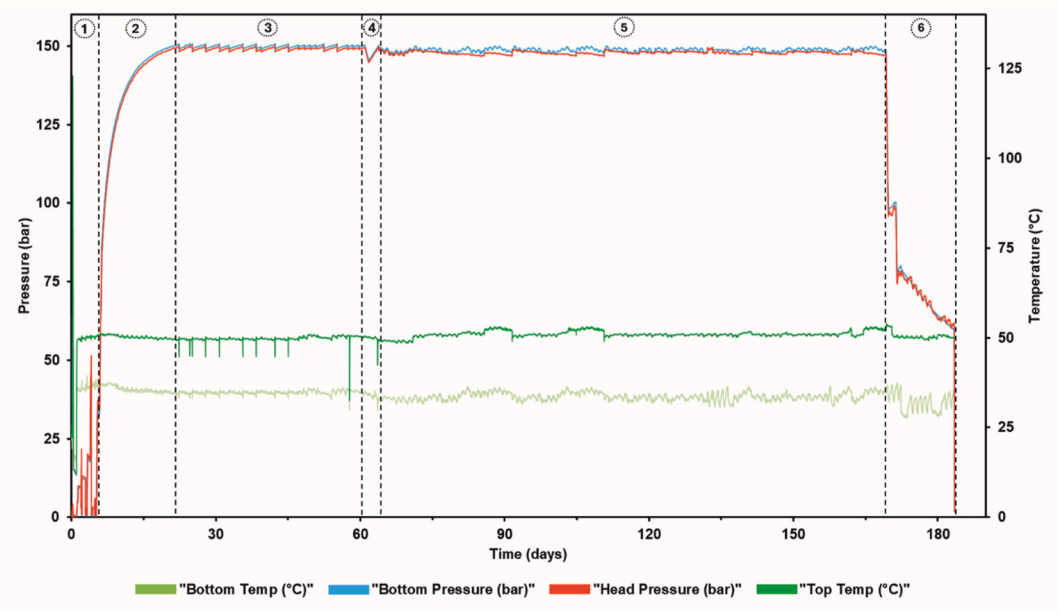

Fig. 3. Trends of Temperatures and Pressures (Headspace & reactor bottom) along R1 test.

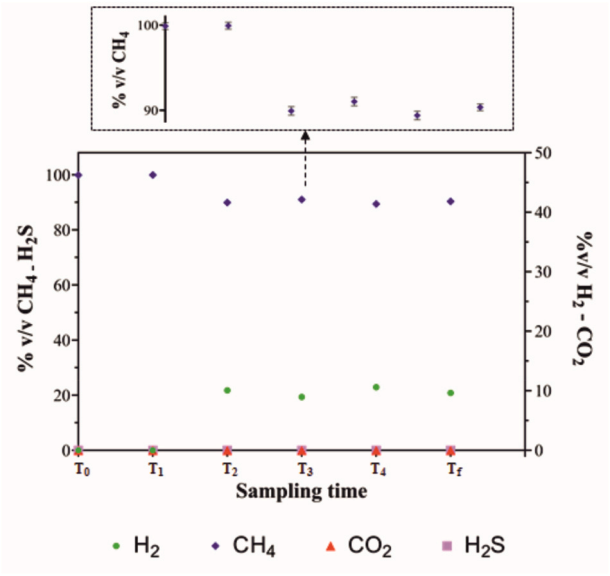

Fig. 4. Trends of CH_4 - H_2 – CO_2 – H_2S volumetric compositions along R1 test with a detail on the CH_4 values.

situation. Indeed, after 6 months of incubations it was observed that the rock samples incubated in the vessels to emulate the solid phase of the reservoir were soaked and moistened by the liquid phase. This may cause the slight increase in the pH value from T_0 to T_f by altering the amount of dissolved carbon, which in turn affects the carbon equilibrium in the liquid phase [41–45]. Hydrochemical analyses carried out at the end of the experiment could validate this hypothesis. Similarly, when the liquid phase was extracted from the reactors, considerable solid debris were observed. This condition can obviously affect the measurement of optical density.

3.1.1. HPLC analysis on VFA

The assessment by means of HPLC of VFAs variations in concentrations have been proven a valuable tool to identify intermediates and substrates of microbial metabolisms, which presence can be addressed to microbial growth and activity [46]. VFAs analysis of formation water samples used as liquid phase during reactor operation was performed on samples collected at both T_0 and T_f to evaluate possible variations in VFAs levels. It was observed that in both samples no trace of VFAs were found almost indicating complete absence of active metabolic pathways (Table 3 and Fig. S6).

Absence of VFAs, which are commonly considered both substrates and products of different microbial metabolisms, is of interest for the present study which aims to evaluate risks posed by presence of HM, SRB and AB during UHS. In anaerobic environments during both acidogenesis and acetogenesis, CO_2 that is used by HM as their sole carbon source is generally produced. Nonetheless, absence of VFAs like acetate, propionate and butyrate which degradation leads to CO_2 generation could potentially cut the carbon supply for HM limiting their growth and activity and potential risks associated to it.

3.1.2. GC analysis of headspace gas phase

Along with the results obtained from HPLC analysis, GC measurement of headspace gases concentrations for R1 did not displayed signs of microbial activation (Fig. 4).

HM and SRB were addressed as possible sources of risk for UHG due to their metabolic requirements for H_2 and respective production of CH_4 and H_2S. Activity of the two microbial clusters would cause consumption of H_2 causing decrease of its concentration in headspace gases, whilst increasing CH_4 and H_2S levels. Monitoring of headspace gas concentrations between T_2 and T_f revealed that the H_2:CH_4 ratio of R1 (90:10) remained stable and unvaried with no detectable traces of the two microbial metabolites. Furthermore, in accordance with results of VFA analysis, no trace of CO_2 derived from acidogenic and acetogenic processes was detected.

As is evident from the results, despite for the T_1-T_2, in which we change the composition by adding the H_2, the changes in the gas composition are almost negligible along all the testing time. Variations are at most in the range of 1–2% and are mainly due to the gas-liquid dissolution equilibria and slight temperature/pressure changes which occur during long experimentation, as reported for the pressure in Fig. 3.

3.1.3. qPCR analysis

Along with the O.D., VFA and GC measurements, qPCR analysis allowing enumeration of specific classes of microorganisms by targeting organism specific DNA sequences were deployed. In this analysis, qPCR methodologies targeting mcrA and dsrB genes coding for key enzymes of methanogenic and sulphate reducing pathway were employed for the enumeration of HM and SRB during batch cultivation of formation water in the bioreactor. Obtained results provided valuable information regarding the possible growth of both groups of microorganisms. In the present study, analysis of samples collected upon reactor inoculation evidenced complete absence of dsrB sequences indicating absence of SRB. HM presence was reported in both rocks and fluid samples and the values of mcrA copies/ml were in the same order of magnitude for both rocks (5.21×10^3 copies/ml) and liquid (4.10×10^2 copies/ml).

After 6 months of incubation, DNA was extracted from both rock and liquid samples collected from R1 and qPCR analysis was performed. Absence of SRB was confirmed with dsrB copies/ml being reported far below the threshold of detection of the devised qPCR methodology for both rock and liquid samples ($<1.33 \times 10^2$ copies/ml). For what concerns HM, a marked decrease, slightly far from the threshold of detection, was observed in mcrA copies/ml detected in both rock samples and liquid samples copies/ml of the targeted gene.

The absence of SRB in the present study is of considerable importance due to the fact that their metabolism may lead to both H_2 consumption and H_2S generation that could induce reservoir acidification and material damage during UHS [47–51]. The absence of such group of microorganisms, as evidenced by qPCR analysis, the risk that could be associated to their activity and growth would be null.

Although only few works report testing of microbial consortia from geological formation by emulating reservoir conditions in reactor systems, results obtained from reservoir A formation fluids differ from those reported in the literature. In fact, Both Haddad et al. (2022) [52] and the Underground SUN Storage project (2017) [32] evidenced HM activity during their tests. When considering HM, the above mentioned studies clearly links HM growth and activity to the presence of CO_2 in tested gas mixtures. When testing formation fluids from reservoir A, CO_2 was not injected in the reactor or generated by other microbial processes. On this based, it is likely that the methanogenic activity leading to the detected reduction in copies/ml of marker gene mcrA has been limited.

Results displayed by both qPCR, VFA and GC analysis here presented provide a valuable scenario regarding the potential risk posed by both HM and SRB for the H_2 underground storage in environmental conditions similar to the one reported for reservoir A. On the one hand, we found no molecular or analytical evidence of the presence of metabolically active SRBs, sulphate content in RA formation water setting to 0.13 g/L, and this finding, on its own, excludes the risk of microbial reduction of sulphate compounds. On the other hand, the possible activation of detected HM is severely limited by the lack of nutrient substrates and carbon sources available to archaea populations. Moreover, although during UHS, the H_2 injected in high amount could be used as reducing power for HM, the observed low microbial activity of HM natural symbionts (i.e. acidogenic and acetogenic organisms) could further reduce the availability of CO_2, limiting HM growth and hence H_2 consumption [53–56].

3.2. R2 testing campaign: reservoir B, 99%H_2:1%CO_2

The formation water of reservoir B was characterized by lower salinity and higher content of carbon sources (i.e. bicarbonates, TOC, **Table 2**) respective to reservoir A. The formation fluids and rocks from reservoir B were tested in the reactor fed-batch experiment in order to evaluate possible microbiological effects on the H_2:CO_2 mixture (99:1 ratio) at reservoir temperature and pressure also during the incubation phase.

During reactor operations constant monitoring of temperatures and pressures was performed to ensure that operational conditions were constantly within those reported for reservoir B. Both T and P were reported to be constant enough during the 6 months of batch operations (variations in the range of 2 %), confirming correct reactor operations with minor technical adjustment, not interfering with operations, as reported in Fig. 5.

As for R1, the trends of temperatures and pressures along the different operational phases are reported for R2 test in Fig. 5 as follows:

- Phase 1: Sanitization – Sterilization – Inoculation (2 days)
- Phase 2: CH_4 and formation liquid feeding (4 days);
- Phase 3: maintenance with CH_4 at high pressure (2 months);
- Phase 4: change of gas composition from 100%CH_4 to 99%H_2: 1% CO_2 (3 days);
- Phase 5: maintenance at high pressure with gas mixture (4 months);
- Phase 6: end of test, sampling of gas/liquid/rock (5 days).

Monitoring of operational parameters (O.D., pH), scVFA concentrations and variations in the stored gas mixture are displayed in Table 4.

During the whole time of the experimental monitoring of O.D., it did not reveal any changes in the turbidity of formation water, whilst a gradual increase in pH from 6.2 to 6.6 was reported between T_0 and T_f.

3.2.1. HPLC analysis on VFA

HPLC analysis revealed acetate as the most represented VFA species which is increasing from 53 to 60.3 mM between T_0 and T_3 with levels of the acid decreasing to 54 mM by the end of 6 months of operations (T_f). Conversely, levels of both lactate and propionate were reported stable between 1.07-1.17 and 0.45–0.88 mM, respectively. The presence of different VFAs species is of importance when considering possible microbial activation, due to the fact that different microbial species (AB) can use both acetate and lactate as main substrates for their metabolisms [57]. Nonetheless, with levels of the acids being reasonably stable during the whole length of the experiment we could assume that both biological uptake and generation of the two chemical species appear to be limited.

Table 4
Operational conditions and experimental parameters monitored for the liquid phase at the beginning (T_0), during the incubation (T_2 and T_3) and end (T_f) of R2 operations used to evaluate activation of the microbial population.

Sampling time (listed in Table 1)		T_0	T_2	T_3	T_F
Reactor - H_2:CH_4 Ratio (%/%)		R2 - 10:90			
Operational T(°C)		50			
Operational P (bar)		150			
Liquid sampling volume (ml)		50			
Liquid flow rate for fed-batch operation (ml/min)		0.1			
pH		6.2 ± 0.2	6.35 ± 0.04	6.36 ± 0.06	6.6 ± 0.01
O.D. (600 nm)		0.00	0.00	0.00	0.00
VFA (mM)	Lactate	0	1.173	1.173	1.068
	Acetate	53.03	58.81	60.433	54.661
	Propionate	0.45	0.8815	0	0.79
mcrA (copies/ml)	Fluid	$4.27 \times 10^3 \pm 1.17$	$4.58 \times 10^3 \pm 1.89$	$1.53 \times 10^2 \pm 2.52$	$1.29 \times 10 \pm 1.29$
dsrB (copies/ml)	Fluid	$5.76 \times 10^2 \pm 1.7$	$5.63 \times 10 \pm 1.05$	$8.16 \times 10 \pm 1.08$	0

3.2.2. GC analysis of headspace gas phase

For what concerns concentrations of the injected H_2:CO_2 gas mixture, during the 4 months of incubation a slight increase (1 ± 0.1 % to 1.5 ± 0.25 %) in the level of CO_2 was reported in the last month between T_4 and T_f (Fig. 6). Such small variations could be attributed to the reduction in acetate levels observed between T_3 and T_f (Table 4). In

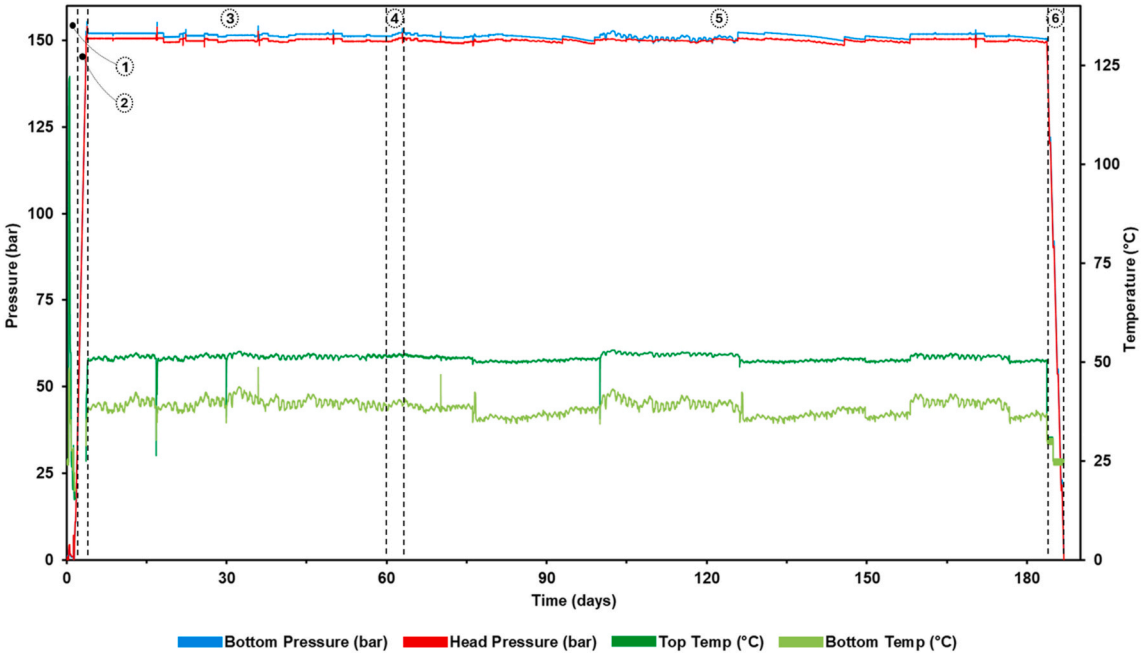

Fig. 5. Trends of Temperatures and Pressures (Headspace & liquid) along R2 tests.

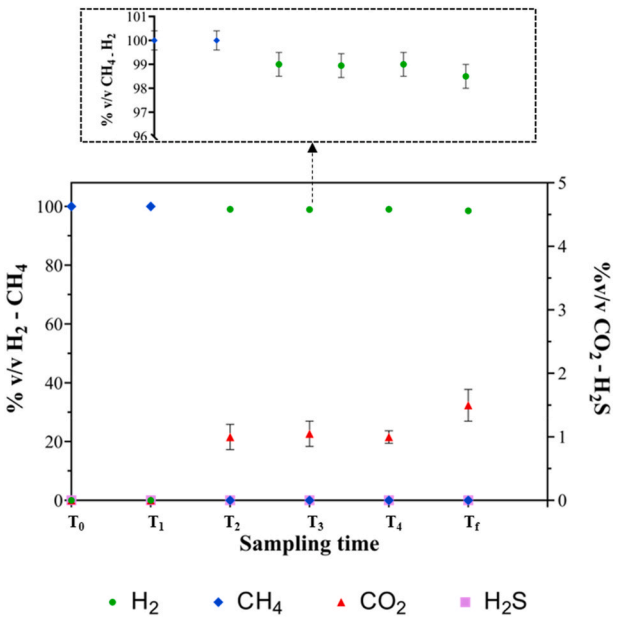

Fig. 6. Trends of CH_4 - H_2 – CO_2 – H_2S volumetric compositions along R2 test with a detail on the CH_4 - H_2 values.

fact, different microorganisms belonging to the *Clostridiales*, *Synergistales* and *Thermotogales* orders can metabolize acetate, generating CO_2 [46,51, 58–60].

Although some of the main substrates for both HM (i.e. H_2 and CO_2) and SRBs (i.e. lactate, acetate, H_2 and SO_4^{2-}) are present in the inoculated formation water, no traces of their main metabolic products, namely CH_4 and H_2S, were detected in the gas phase of the system evidencing no activity of the two microbial clusters.

3.2.3. qPCR analysis

To verify this assumption, qPCR analyses were performed on reactor formation fluid by targeting functional genes involved in HM and SRBs metabolisms (Table 4). The variations in population density (copies/ml) for HM (mcrA) and SRB (dsrB) during 6 months of operations were detected. Samples were collected at the time sampling reported in Table 1.

Initial characterization of reservoir B formation water evidenced low density of both HM and SRB populations with copies/ml for mcrA and dsrB reported at 4.27×10^3 and 5.76×10^2 respectively.

Population density of the two microbial clusters remained somewhat stable during adaptation in CH_4 (T_0- T_1), although from the moment of the gas shift and injection of the H_2:CO_2 blend a considerable decrease in copies/ml for both targeted genes was reported. By the end of the 4-month incubation with H_2:CO_2 (T_f), copies/ml of both mcrA and dsrB were reported below their threshold of detection. Results of qPCR analysis clearly evidenced the presence of both HM and SRB in reservoir B formation water at T_0- T_1, nonetheless, the reported dynamic for HM and SRB seems to address considerable limitations to their proliferation and activity in reservoir conditions.

Along with the absence of HM and SRBs metabolic products (i.e. CH_4 and H_2S) in the gas phase, the reduction of copies/ml of specific marker genes (i.e. mcrA and dsrB) allows defining a low level of biological risk posed to possible UHS procedures by reservoir B indigenous population.

For what concerns SRBs possible causes of reduced activity, it could be addressed by the already low SRBs population density, and by the low levels of sulphate present in formation waters (Table 2).

The literature reports SRBs as capable to grow between sulphate concentrations of 14–300 mg/l [32] . In Haddad et al. (2022) [52] during reactor experiment emulating reservoir conditions, although SRB densities in initial inoculant appears to be higher than those of reservoir B. Indeed (Haddad et al., 2022), detected the marker gene dsrB between 10^4 and 10^5 copies/ml whilst the initial dsrB density of reservoir B in the present work is 5.76×10^2 copies/ml.

Due to their metabolic flexibility (stams plugge, 2008), one could hypothesize that during the adaptation phase of resevoir B, the absence of reducing power (H_2) shifted SRB metabolism towards chemo-organotrophic pathways resulting in competition for substrates with acidogenic and acetogenic bacteria, commonly present in fluids from underground environments. This along with the low density of the SRB population of reservoir B could have led to the drop observed between T_0 and T_F (Table 4).

Conversely, HM were provided with a considerable amount of both reducing power (i.e. H_2) and carbon sources (i.e. CO_2, HCO_3^-) for their metabolism, nonetheless population density declined during the experiment. One possible reason for such decline might be attributed to substrate competition with other microbes inhabiting the reservoir (i.e. acetogenic Clostridia), a phenomenon which normally occurs in the natural and anthropic environment [53,61]. This hypothesis might be supported by the presence of VFA traces (Acetate, Propionate, Lactate) in the liquid phase, which are typically linked to acetogenic metabolism (AB). Acetate, in particular, slightly increases between T_0 and T_3, potentially indicating a metabolic activity related to AB. Alternatively, another possible hypothesis could be that the exposure to H_2 and CO_2 mixture did not lead to the proliferation of homoacetogenic bacteria and hydrogenotrophic methanogens, which instead dropped in relative abundance. This outcome suggests that, despite the provision of 1% CO_2 in the gas mixture and the higher carbon and bicarbonate content present in reservoir B formation water, compared to reservoir A, the NaCl levels and the slight acidic pH (Table 2), may attribute to reservoir B microbiome a low ability to adapt to conditions other than the natural reservoir conditions (i.e., injection of different gas mixture and variation of pressure).

4. Conclusions

In conclusion, this study presents an innovative approach to assess the microbiological risks associated with UHS using an innovative customized reactor system, the Bio-xplorer. The design and implementation of the experimental set-up applied in the present study allowed for a comprehensive analysis of UHS key factors and helped the evaluation of the potential microbial activity in two Italian reservoirs, named Reservoir A and B.

The experiments included the monitoring of pressure, temperature, cell optical density, pH, volatile fatty acids (VFAs) concentrations, and microbial density using quantitative polymerase chain reaction (qPCR). Furthermore, gas chromatography analysis provided insights into changes in the gas mixture composition. The use of the Bio-xplorer provided a comprehensive understanding of the microbial dynamics and interactions through an accurate simulation of the UHS environment of those reservoirs. In particular, in Reservoir A, the stable pressure and temperature trends, low optical density readings, absence of VFAs, and lack of detectable microbial metabolites in the gas phase indicated minimal microbial activation. Similarly, in Reservoir B, the absence of sulphate-reducing bacteria (SRB) activity and the decline in hydrogenotrophic methanogens (HM) density suggested a reduced biological risk.

The results obtained from the experiments demonstrated the effectiveness of the designed system in assessing the microbiological risks associated with UHS and highlighted the importance of considering reservoir-specific conditions and microbial activity when assessing the long-term effects of UHS on possible hydrogen conversion.

The knowledge gained from this study contributes to the safe and efficient implementation of UHS projects, emphasizing the need for risk assessment and mitigation strategies. Furthermore, the innovative reactor system presented in this study serves as a valuable tool for exploring in the first place and then predicting the evolution of the

microbial population, the identification of potential hazardous biogenic molecules, and the development of appropriate monitoring and control strategies. Further research will be carry out in order to validate these findings, also in other potential UHS reservoirs, and to investigate additional factors, e.g. temperature, pH and salinity, that may affect microbial activity in UHS environments. Moreover, a reactor testing campaign using the same formation fluids form reservoirs A and B will be carried out at the same operative conditions in not limiting nutrient condition. By supporting the advances in the understanding of microbial processes in UHS, we can ensure the future safe and sustainable utilization of hydrogen as an energy storage solution.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: With respect to potential conflicts of interest, we hereby declare that Christian Coti, Donatella Barbieri and Matteo Scapolo are employed by Snam Stogit S.p.A., a company specialized in geological natural gas storage.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhydene.2023.10.245.

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