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1 **Biological, Geological and Chemical Effects of Oxygen Injection**
2 **in Underground Gas Storage Aquifers in the setting of**
3 **Biomethane Deployment**

4
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24

25 **Abstract**

26 The last few years have seen the proliferation of anaerobic digestion plants to produce
27 biomethane. Oxygen (O₂) traces added to biogas during the desulfurization process are co-
28 injected in the gas network and can be stored in Underground Gas Storage (UGS). However,
29 there are no data available for the undesirable effects of O₂ on these anoxic environments,
30 especially on deep aquifers. In addition to mineral alteration, O₂ can have an impact on the
31 anaerobic autochthonous microbial life. In our study, the storage conditions of an UGS
32 aquifer were reproduced in a high-pressure reactor and bio-geo-chemical interactions between
33 the aqueous, gas and solid phases were studied. Sulfate was depleted from the liquid phase for
34 three consecutive times during the first 130 days of incubation reproducing the storage
35 conditions (36 °C, 60 bar, methane with 1% CO₂). Sulfate-reducers, such
36 as *Desulfovibrionaceae*, were identified from the high-pressure system. Simulations with
37 PHREEQC were used to determine the thermodynamic equilibrium to confirm any gas
38 consumption. CO₂ quantities decreased in the gas phase, suggesting its use as carbon source
39 by microbial life. Benzene and toluene, hydrocarbons found in traces and known to be
40 biodegradable in storages, were monitored and a decrease of toluene was revealed and
41 associated to the *Peptococcaceae* family. Afterwards, O₂ was added as 1% of the gas phase,
42 corresponding to the maximum quantity found in biomethane after desulfurization process.
43 Re-oxidation of sulfide to sulfate was observed along with the end of sulfate reducing activity
44 and toluene biodegradation and the disappearance of most of the community. H₂ surprisingly
45 appeared and accumulated as soon as hydrogenotrophic sulfate-reducers decreased. H₂ would
46 be produced *via* the necromass fermentation accomplished by microorganisms able to resist
47 the oxic conditions of $4.42 \cdot 10^{-4}$ mol.Kgw⁻¹ of O₂. The solid phase composed essentially of
48 quartz, presented no remarkable changes.

49

50 **Keywords:** deep aquifers, biomethane, oxygen, deep subsurface, high pressure, UGS,
51 geological storage

52

53 **1. Introduction**

54 Oxygen (O₂) can be injected in deep underground for many purposes. Its ability to promote
55 aerobic microbial metabolism is a key factor to achieve the endpoint in multiple current
56 practices especially during the application of enhanced oil recovery techniques on oil
57 reservoir (Cai et al., 2015; Liu et al., 2018; Pannekens et al., 2019) and the bio-remediation of
58 groundwater (EPA, 2017; Lien et al., 2016; Trulli et al., 2016). However, despite its
59 aforementioned beneficial applications, the presence of oxygen can be deleterious during
60 other procedures such as Carbon Capture and Storage (CCS) (Morgan et al., 2017; Wei et al.,
61 2015), compressed air energy storage (CAES) (Guo et al., 2021; Kushnir et al., 2012) and
62 underground natural gas storage (UGS). Many studies have been undergone in the context of
63 CCS storage in order to define the effect of carbon dioxide, but also the effect of oxygen
64 injected as an impurity on the geological formations. Similarly, the deep underground storage
65 of biomethane can also involve co-injection of O₂. Indeed, O₂ is added to the biomethane
66 during sulfide reoxidation at the outlet of the anaerobic digestion plant. Depending on the
67 initial sulfide quantity, the proportion of O₂ may attain 1.5 % of the final gas mixture (Barik et
68 al., 2013). For underground gas storage, France sets a maximal O₂ proportion of 100 ppm,
69 whereas Germany sets a maximal of 10 ppm.

70 Biomethane is an attractive alternative to fossil fuels particularly because biogas produced
71 from biomass, such as green waste and sludge from wastewater, prevents re-circulating
72 carbon from oil, natural gas and coal. With a reported production of 1.2 billion m³ in 2015,
73 Europe is the world's leading biomethane producer. The lead application of the produced
74 biomethane consists of its use as vehicle fuel or its injection into the natural gas grid (Scarlat
75 et al., 2018). With the fluctuating and unpredictable demand throughout the year, biomethane
76 storage is recently gaining an increasing interest. Due to their large storage capacity and their
77 ability to preserve gas under pressure, deep aquifers emerge as attractive underground storage

78 systems. Deep Underground Gas Storages (UGS) such as deep aquifers, are known for being
79 anoxic environments that form large ecosystems and harbor a much-diverse anaerobic
80 microbial life (Magnabosco et al., 2018). The biodegradation of hydrocarbons was notably
81 studied in this setting, especially the anaerobic degradation of BTEX (Benzene, Toluene,
82 Ethylbenzene and Xylenes isomers) by autochthonous microorganisms in these ecosystems
83 (Aüllo et al., 2016; Bombach et al., 2010; Ranchou-Peyruse et al., 2017). Sulfate-reducing
84 bacteria (SRB) are particularly known to be active and to be key players in such anoxic
85 environments (Berlendis et al., 2010; Bombach et al., 2010; Detmers et al., 2004; Fry et al.,
86 1997; Itävaara et al., 2011; Ranchou-Peyruse et al., 2019). Part of the O₂ co-injected with the
87 biomethane during its storage can solubilize into the formation water leading to alterations of
88 the preexisting thermodynamic equilibrium, which can result in drastic modifications in the
89 microbial communities and depletion of strictly anoxic micro-organisms. O₂ may also cause
90 mineral oxidation of the reservoir rock (Guo et al., 2021; Jung et al., 2013; Kushnir et al.,
91 2012; Lu et al., 2016; Vu et al., 2018). For instance, the oxidation of pyrite (FeS₂) was
92 observed during CAES in different studies (Guo et al., 2021; Kushnir et al., 2012) and during
93 CCS (Jung et al., 2013; Shao et al., 2014). This alteration can cause a variation in the
94 reservoir's porosity and permeability especially if large amounts of redox-sensitive minerals
95 were present (Lu et al., 2016). Dissolution of rocks, mineral precipitations and corrosion of
96 well materials were shown as possible (Zettlitzer et al., 2010; Wang et al., 2011). Variation in
97 the aqueous phase composition resulting from mineral dissolution can impact the
98 thermodynamic equilibrium. Identifying this equilibrium is important to determine the
99 material balance and to estimate any production or consumption of gas such as carbon dioxide
100 consumption by the microbial life as a carbon source or oxygen consumption due to chemical
101 reactions. However, experimental solubility data corresponding to our aqueous and gas
102 compositions under aquifer's conditions are very limited. The studied conditions of the

103 system were a temperature of 36 °C and a total pressure of 60 bar, similar to the aquifer's real
104 storage conditions. Even though many CCS studies targeted storage effects on the site,
105 multiple uncertainties remain regarding the effects of impurities namely O₂ during
106 biomethane storage in natural gas reservoirs such as aquifers. In order to elucidate more
107 answers on this topic, a multi-disciplinary study is undergone and, to our knowledge, this is
108 one of few studies involving the reproduction of the aquifer's *in-situ* conditions in a reactor
109 using solid, liquid and microbial underground samples. During this study, the aqueous and gas
110 compositions were followed up regularly *via* chromatography techniques. Samples from the
111 aqueous phase were recovered under anoxic conditions for microbial studies. Afterwards, O₂
112 was injected at 1% of the gas mixture, corresponding to the maximum quantity found in
113 biomethane. Before and in the end of the experiment, the solid phase composed of a reservoir
114 rock sample undergone mineral analyses. The subsequent bio-geo-chemical interactions
115 between the gas mixture, reservoir rock, formation water and harbored microbial life were
116 determined under temperature and pressure of a selected aquifer UGS.

117

118 **2. Materials and methods**

119 *2.1- Site description and formation water characteristics.*

120 The studied reservoir is a UGS in the South Aquitaine sedimentary basin (582 m depth), in
121 Southwest France used for natural gas storage since 1981. The well that was selected for this
122 study is the closest to the gas bubble (about 250 m) and has already been identified in
123 previous studies with the code name Ab_L_1 (Ranchou-Peyruse et al., 2019). Thus, sampled
124 water is the most exposed for gases interactions due to its proximity to the gas bubble. Some
125 of the physicochemical characteristics of the formation water (LCE-SOBEGI Laboratory,
126 Lacq, France) and its overall composition were summarized in Table 1. Formation water

127 (0.634 L) was recovered from the well under pressure (January 2019) with a downhole
128 sampler, equipped with a PDS Sampler (Leutert Bottom Hole Positive Displacement Sampler)
129 (Kampman et al., 2014). Wellhead water samples were also recovered. Samples were stored at
130 4°C until use. Wellhead samples were filter-sterilized at 0.1 µm (PES 47 mm membranes,
131 0.1 µm, Sartorius) while maintaining anoxic conditions: 0.854 L of these samples was added
132 to the formation water (0.634 L) to increase the volume, a month before the injection into the
133 high-pressure reactor. A volume of 0.1 L was taken from the final mixture for a microbial
134 diversity study, whereas the rest (1.39 L) was injected into the reactor.

135 The reservoir rock was composed of infra-molassic sands, with a porosity varying between 25
136 and 35 % (Ranchou-Peyruse et al., 2019). The rock samples were composed of cuttings
137 collected by TEREGA from a drilling carried out near the studied site. X-ray diffraction
138 analysis applied on the solid sample before the experiment showed that the solid phase was
139 mainly composed of quartz with minor calcite content (Figure S1). Iron sulfides (pyrite and
140 marcasite), iron oxi-hydroxides (maghemite and hematite) and clay minerals were also
141 identified as trace minerals.

142 *2.2- High-pressure reactor.*

143 The apparatus was composed of an experimental cell presented in Figure 1. The autoclave
144 was made of Hastelloy C-276 in order to prevent any corrosion problems. A basket held the
145 solid phase and was composed of a metallic disc with pores of less than 10 µm in order to
146 prevent particles from falling to the bottom of the reactor without interfering with the
147 movement of microorganisms. The operating pressure and temperature can go up to 150 bar
148 and 150 °C respectively. A safety valve of 150 bar connected to a pressure compensation
149 chamber protected the overall system. An insulating coating with heating resistors heated the
150 autoclave. Two thermocouples permitted monitoring of the liquid and gas phase temperatures
151 with a precision of ± 1 °C. A double jacket was used to maintain the temperature of the

152 experimental cell. The cell pressure was monitored by the Keller PA(A)-33X pressure gauge
153 with a precision of 0.3 bar. In order to maintain a constant pressure throughout the
154 experiment, a mobile piston was placed into the reactor. A 1L capacity pump allowed the
155 piston to move upwards in order to increase the cell pressure and downwards to decrease it.
156 The pump can operate at a maximum pressure of 150 bar and was monitored by a pressure
157 gauge with a precision of 0.05 %. A flow rate of up to 220 mL/min can be imposed with a
158 precision of 0.1 % of the set point. The overall autoclave volume was 5.41 L with an available
159 volume of 4.2 L when the piston was at its lowest level. The solid basket had a volume of 0.8
160 L. The system was mixed using a stirring shaft operated by a variable speed motor with a
161 maximum stirring speed of 500 rpm. A Rushton turbine was used for the liquid phase and a
162 double disc stirrer with four vertical blades for the gas phase. The composition of the gas
163 mixture injected at the beginning of the biotic experiment was methane, 1 % of carbon
164 dioxide, 7.95 ppm of benzene and 3.57 ppm of toluene. The oxygen added to simulate
165 biomethane injection had a purity of >99.995% (CAS: 7782-55-7).

166 The experiment ran over 180 days. Initially, a volume of 1.39 L of aqueous phase was
167 injected in the reactor and $4.57 \pm 2.2 \cdot 10^{-1}$ mol of gas mixture. The system liquid-gas-solid-
168 biomass was under development for 130 days under a total pressure of 60 bar and a
169 temperature of 36 °C. The oxygen injection (1 % of 60 bar) occurred at day 131. The Figure 2
170 presents the analysis undergone through the experiment at different timings.

171 *2.3- Abiotic experiment*

172 A preliminary abiotic experiment was initially undergone in order to detect the liquid-solid
173 interactions before adding the microbial life to the system. 500 mL of distilled water were
174 mixed with 100 mL of sand reservoir in a sealed 1L bottle that was subsequently flushed with
175 N₂ gas at atmospheric pressure. The composition of the aqueous phase was followed in order
176 to detect compounds dissolution from the solid into the liquid phase.

177 *2.4- Analytical analyses.*

178 The liquid phase was analyzed using an ionic chromatography with a variable wavelength
179 detector from ThermoFisher Scientific (Dionex Integrion HPIC). The column IonPac AS28-
180 Fast-4 μ m (4x150 mm) was used with the AG28-4 μ m (4x30 mm) guard-column for anion
181 detection, particularly fluoride, chloride, sulfate, acetate, carbonate/bicarbonate and sulfide.
182 The cations were quantified with the Dionex Easion System (ThermoFisher Scientific). The
183 column IC Dionex IonPac CS12A (4x250 mm) was used with the guard-column CG12A
184 (4x50 mm) for sodium, potassium, magnesium and calcium. A liquid sample was taken from
185 the liquid output using a syringe and stored in a flask that was sealed with rubber. Air was
186 immediately extracted from the flask using N₂ gas flush. The sample (1 mL) was directly
187 injected into the instrument. The analyses were performed in triplicate. The supposed
188 measurement uncertainty on both techniques was $\pm 5\%$.

189 The gas phase composition was monitored by a gas chromatography in line with a micro
190 thermal conductivity detector (GC- μ TCD; Micro GC Fusion; Chemlys; France). The
191 'Molsieve 5A' column was used with helium as a carrier gas for quantification of methane,
192 hydrogen, oxygen and nitrogen. The column 'Plot Q' was used with argon as carrier gas for
193 quantification of carbon dioxide and hydrogen sulfide. The instrument was connected to the
194 reactor's valve. Once open, the sample traversed a pressure compensation tube to reach the
195 piston where it attained the atmospheric pressure and it undergone analysis at that moment.
196 All measures were obtained after three analyses, and the uncertainty was $\pm 2\%$. The obtained
197 results were used to calculate the quantity of each component in the gas phase. The non-ideal
198 gas equation was used to calculate the total number of moles in the autoclave at the moment
199 of analysis. The selected compressibility factor was 0.9 (calculated with PHREEQC using
200 phreeqc.dat as database).

201 The identification and quantification of benzene and toluene in the liquid and gas phases were
202 performed using a ThermoFisher Scientific gas chromatograph coupled with a quadrupole
203 mass spectrometer (GC-MS) (ISQ QD Single Quadrupole MS - Trace 1310).
204 Preconcentration of benzene and toluene was performed by SPME with a
205 polydimethylsiloxane/carboxene (PDMS/CAR) fiber and the chromatographic separation was
206 performed by a DB-624 column (Agilent). For each sample, two 10 mL water samples were
207 taken and stored at 4°C. Prior to the analysis, 90 µL of the 0.5 ppm 1,2,4-trimethylbenzene
208 internal standard were added to each 6.9 g sample. Gas samples were collected using a needle
209 sampling system controlled by a manometer (1bar/vial). Quantification was performed with
210 methane as reference gas containing 10 mol-ppm of benzene and toluene.

211 *2.5- Treatment of the solid phase.*

212 Before the experiment, the reservoir rock was rinsed with isopropanol followed by water in
213 order to eliminate potential hydrocarbons and salts such as calcium chloride used during the
214 drilling operation. After rinsing, the solid sample was dried and sterilized overnight in the
215 oven at 90°C. A sample of 450 mL of the solid phase was placed in the basket inside the high-
216 pressure reactor. Capillaries used for X-ray tomography were also filled with the solid phase
217 and placed in the basket.

218 Once the reactor closed, a volume of 60 mL of demineralized water was injected and a flush
219 of N₂ gas was applied. The autoclave was heated to 100 °C during 24h, water vapor was
220 evacuated through the tubes and valves for sterilization. Afterwards the reactor was cooled
221 down, heated to the desired temperature of 36 °C and the water phase (with autochthonous
222 microorganisms) was injected.

223 After 180 days of experiment at 36 °C and 60 bar, the remaining liquid phase was removed
224 but maintained in sterile and anoxic conditions and was kept at 4°C awaiting further analysis.

225 Afterwards, the solid basket was quickly and carefully removed from the reactor and stored in
226 a sealed anaerobic jar with anaerobiosis generator and indicator pockets (Dutscher Ref
227 0260001) before being transported to an anaerobic glove box.

228 *2.6- Thermodynamic modelling.*

229 The thermodynamic equilibrium at different times of the experiment was calculated using
230 PHREEQC thermodynamic software (Parkhurst and Appelo, 1999). This software allows a
231 comprehensive introduction of dissolved species, solid and gaseous phases and the
232 corresponding chemical reactions. The thermodynamic data were obtained from 'Phreeqc'
233 database without any optimization or modification. Phreeqc.dat uses the extended Debye-
234 Hückel law for activity coefficients and the Redlich-type equation of state for the gas phase.
235 Steady state was assumed for all calculations. All the gas species were considered, but redox
236 reactions of methane and nitrogen were neglected. Water evaporation was considered. Sulfate
237 reduction reaction was not considered in the anoxic part of the experiment. Phreeqc was
238 mainly used to calculate gas solubilities in absence of reactions, thus interactions with the
239 solid phase were not considered. The calculations took into account the different water
240 injections occurring during the experiment and the quantity of gas sampled during the
241 chromatography analysis.

242 *2.7- Cell culture media, strains isolation and brief characterization.*

243 Different synthetic culture media were prepared for the assessment of the microorganisms
244 developed in the reactor just before O₂ addition to the system. In order to prepare these media,
245 a mineral base mimicking the formation water composition was prepared (per liter) by adding
246 0.0002g NH₄Cl; 0.01g KCl; 0.13g CaCl₂·2H₂O, 0.023g MgCl₂·6H₂O and 0.015g K₂HPO₄, 10
247 ml of trace elements solution (SL12; (Eichler and Pfennig, 1986)) and 0.5 mL of selenite
248 tungstate solution (Widdel and Bak, 1992). One gram of cysteine as reducing agent and 1 mL

249 resazurine (0.1% solution, w/v) as redox indicator were supplemented. Depending on the
250 targeted metabolic groups, other compounds were also added. For fermentative bacteria, 10g
251 yeast extract; 2.5g glucose; 15g casein peptone were added whereas for sulfate-reducers,
252 0.82g sodium acetate; 0.5g yeast extract; 0.68g sodium formate; 1.1g sodium pyruvate; 2.84g
253 sodium sulfate were added. Media were autoclaved at 110°C for 30 minutes and then cooled
254 down at room temperature under a stream of N₂. At room temperature, 0.2g of NaHCO₃
255 prepared in anoxic conditions and autoclaved separately from the medium was added to each
256 culture medium. Five milliliters of filtered anoxic V10 vitamins solution (Pfennig et al., 1981)
257 were added. Media were adjusted to a pH of 6.7 and distributed anoxically in Hungate tubes.
258 Enrichment cultures were incubated at 36 °C. The growth was followed by optical density at
259 600 nm (Spectrophotometer, Spectronic Instruments, Spectronic 401).

260 Several strains were isolated from the last tube showing growth during the Most Probable
261 Number technique. The 15A and Recti_s6 strains were thus isolated from the medium
262 targeting the sulfate-reducers, whereas the 2A strain was isolated from the medium dedicated
263 to the fermentative metabolic group. Isolation of each strain was carried out by the method of
264 dilution series in deep agar (Pfennig and Trüper, 1992). Rifampicin was added at a
265 concentration of 10 µg/mL during the Recti_s6 isolation (Koelschbach et al., 2017). The
266 purity of the isolated strains was checked under a phase contrast microscope (BX60;
267 Olympus) as well as by sequencing their 16S rDNA genes. For the fermentative strains (2A &
268 Recti_S6), searching for the anaerobic fermentation products was conducted using 10 mM of
269 glucose and a N₂ gas phase (1 bar). The gas phase was followed by GC-µTCD (Micro GC
270 Fusion; Chemlys; France). The fermentation products were analyzed using ion
271 chromatography (ThermoFisher). This process was conducted at the end of the exponential
272 growth curve, by taking 0.5 mL of culture and filtering it at 0.22 µm (IC Millex®, Millipore)
273 in a sterile degassed Hungate tube. Sterile physiological water was added afterwards in order

274 to reach a final volume of 2 mL. For the sulfate-reducing strain (15A), the experiments were
275 conducted in 120 mL penicillin flasks. The strain was cultivated with H₂/CO₂ (80/20, 1 bar) as
276 energy and carbon sources respectively, in addition to 20 mM of sulfate as terminal electron
277 acceptor. The final products obtained from the sulfate consumption by this strain were
278 monitored in these conditions, then with 10 mM of formate as carbon and energy sources and
279 20 mM of sulfate under N₂ atmosphere. The changes in the gas and liquid phases were
280 investigated by gas micro-chromatography and ion chromatography, respectively. The
281 microbial growth was followed by optical density at 600 nm as described above. The isolated
282 strains were tested for their oxygen tolerance. Different volumes of air, filtered at 0.1 μm,
283 were added to several Bellco tubes. A tube with a nitrogen gas phase and another one
284 containing only air served as controls. The gas phase was analyzed at the beginning and at the
285 end of the experiment by a microthermal conductivity detector (Gc-μTCD; MicroGC Fusion;
286 Chemlys; France). The microbial growth was followed by optical density at 600 nm.

287 *2.8- Nucleic acids extraction and RNA retro-transcription.*

288 At different times of the experiment, samples from the liquid phase were collected in order to
289 co-extract the nucleic acids. The samples were directly filtered under vacuum using a 47 mm
290 PES membrane filters of 0.1 μm porosity (by Sartorius Stedim). Afterwards, the collected
291 filters were stored at -80 °C in order to protect the RNA. Filters were crushed in liquid
292 nitrogen and the nucleic acids were extracted using the Fast RNA Prosoil Direct kit (MP
293 BIO). DNA was separated from RNA with All Prep RNA/DNA (Qiagen) kit. From the
294 isolated strains, 2 mL of each culture were pelleted by centrifugation at 10·000 g for 5
295 minutes. The DNA was then extracted using DNeasy Power Soil (Qiagen) kit. After
296 extraction, DNA and RNA were quantified using the Quant-it™ dsDNA HS (Invitrogen) and
297 the Quant-it™ RiboGreen (Invitrogen) kits, respectively. The measurements were done using
298 a BioTEK SYNERGY HTX microplate reader. The reverse transcription of the RNA was

299 performed *via* retro transcriptase M-MLV (Invitrogen™) according to the supplier's
300 recommendations.

301 2.9- Polymerase chain reaction and sequencing.

302 The V3-V4 regions of the 16S rRNA gene of the genomic DNA and cDNA were amplified by
303 nested PCR as previously described (Yu et al., 2015). The search for archaeas was carried out
304 by targeting successively the 21F-958R and 349F-806R regions of the Archean 16S rRNA *via*
305 a nested PCR (Takai and Horikoshi, 2000). The Taq PCR Core Kit (Roche) and the 2720
306 Thermal Cycler (Applied Biosystems) were used. The bacterial PCR primer pairs (344F-
307 801R) that were used contained CTTTCCCTACACGACGCTCTTCCGATCT (forward) and
308 GGAGTTCAGACGTGTGCTCTTCCGATC (reverse) adapters. Afterwards, high-throughput
309 sequencing conducted by the GenoToul genomics platform (Toulouse, France), using
310 Illumina's MiSeq 2x250bp technology in accordance with the manufacturer's instructions was
311 undergone. Sequencing data were processed *via* FROGS analysis pipeline developed by the
312 GenoToul genomics platform in the Galaxy interface (Escudié et al., 2018).

313 Using the DNA of the isolated strains, the 16S rRNA genes amplifications were conducted
314 with the primers set 8F-1489R (Lane, 1991; Weisburg et al., 1991). The amplicons were
315 sequenced by Qiagen Genomic Services (Sanger sequencing; Hilden, Germany).

316 Raw sequences were archived in public NCBI database (SAMN19579666-SAMN19579701).
317 The Genbank/ EMBL/ DDBJ accession numbers of the 16S rRNA gene sequences of isolated
318 strains were: 15A (MZ317661), Recti_s6 (MZ317662) and 2A (MZ317660).

319 2.10- Quantitative PCR.

320 Quantitative PCR (qPCR; Biorad CFX Connect) *via* Takyon NO ROX SYBR 2X MasterMix
321 blue dTTP (Eurogentec) was used to determine the number of 16S rRNA gene copies present
322 in each sample during the incubation at high pressure (from DNA and cDNA). The primers

323 used were 338F (Suzuki and Giovannoni, 1996) and 518R (Muyzer et al., 1993). All reactions
324 had a 20 μ L final reaction volume in accordance with the supplier's instructions. The reactions
325 were denatured at 95°C/3min followed by 40 cycles of 95°C/10sec, 55°C/20sec and
326 72°C/20sec. The number of copies was calculated starting from a standard with serially 10-
327 fold diluted pCRTM 2.1-TOPO plasmid (TOPO TA cloning kit, Invitrogen).

328 *2.11- X-Ray Tomography.*

329 The morphological alterations of the solid phase were followed by X-ray tomography.
330 Capillaries made of borosilicate with an internal diameter of 2 mm and a height of 3 cm were
331 filled with the solid phase (sand) and positioned in the reactor. The bottom of each capillary
332 was blocked with cotton to prevent loss of material. Before the experiment, capillaries were
333 scanned by X-ray tomography for initial reference. After the completion of the experiment,
334 the capillaries were removed and sealed at both ends with waterproof glue and stored at 4 °C
335 in anaerobic glove-box until scanning. A Zeiss Xradia Versa 510 tomograph was used. Based
336 on a prior sensitivity study, the selection of the acquisition parameters was centered on
337 optimizing the image contrast, signal-to-noise ratio and acquisition duration. Typically a set
338 of 1601 radiographies of 1024x1024 pixels with a pixel size of 2.5 μ m was acquired. The X-
339 ray generator was operated at 40 kV and the exposure time of a single radiography is 10s. The
340 set of recorded radiographs was reconstructed with XRM Reconstructor® (Zeiss, version 14)
341 in order to obtain a stack of cross-sections forming a digital volume of the sample. This
342 reconstruction software was based on the standard filtered back projection algorithm. The data
343 were processed, visualized and interpreted using Dragonfly® (ORS, Version 2020.2.0.11731).
344 A filtering step was first applied in order to improve the signal-to-noise ratio of the data. The
345 subsequent segmentation step enabled the extraction of the different phases (solid, liquid,
346 biomass) for further quantitative analyses such as assessment of the proportion and location of
347 each phase in the studied volume and comparison of data sets before and after the experiment.

348 *2.12- X-Ray Diffraction and Scanning Electron Microscopy.*

349 X-Ray diffraction (XRD) was used to study the mineralogy of the reservoir rock. Mineral
350 identification was performed using a Bruker D2 Phaser powder diffractometer with a Cu K α
351 radiation source. XRD patterns were recorded over 5° to 90° 2 Θ with a 0.02° step and a 0.5 s
352 counting time per step. Samples from the reservoir rock were analyzed before the experiment.
353 Bulk rock was analyzed in order to obtain the global mineral phases and a color-based
354 fractionation was performed in order to identify potential minor crystallized phases. After the
355 completion of the reactor experiment, samples from different locations of the basket were
356 collected in the glove box. The collections were performed from three different depths that
357 were 1 cm away from each other. The samples were then dried up under gaseous N₂ in an
358 anaerobic glovebox at ambient temperature. All samples were manually ground and sieved
359 into a homogeneous powder at 100 μ m, the product was then analyzed by XRD. Identification
360 of crystallized phases was done using the DIFFRAC.EVA software.

361 Scanning Electron Microscopy (SEM) was used to localize mineral phases and to detect
362 potential formation of biofilm. Collected samples from three different depths, that were 1 cm
363 away from each other, were immediately fixed in a mixture of cacodylate 0.1 M and 2.5 %
364 glutathione pH 7.2. They were then dried out in successive solutions containing 30 to 100 %
365 ethanol and finally evaporated using hexamethyldisilazane (HMDS). Afterwards, the samples
366 were mounted on stubs and platinum coated. Observations were carried out using a Gemini
367 SEM 300 (Zeiss) combined to Energy Dispersive Analysis (EDX) at 8 kV in secondary
368 electron mode (SE) or backscattering electron mode (BSE).

369

370 **3. Results**

371 *3.1- Monitoring of the physico-chemical evolution during abiotic experiments.*

372 After approximatively 10 days of mixing the liquid and the solid phases, water composition
373 showed an increase of chloride (3.05 ± 0.15 mM), sulfate (0.29 ± 0.01 mM) and calcium (2.18
374 ± 0.22 mM) concentrations in the water, indicating that the mineral matrix released these
375 ions. Very limited quantity of acetate appeared in the liquid phase (0.005 ± 0.0003 mM). The
376 solubilities at the thermodynamic equilibrium of the system liquid – solid – gas were
377 calculated according to the measured variations in the liquid phase. Under a total pressure of
378 60 bar, a temperature of 36 °C and with a gas composition of 98.3% CH₄, 0.8% CO₂ and 0.9
379 % O₂ at equilibrium, the calculated solubility of CH₄, CO₂ and O₂ using PhreeqC and
380 phreeqc.dat are $5.96 \cdot 10^{-2}$, $8.88 \cdot 10^{-3}$ and $5.25 \cdot 10^{-4}$ mol.Kgw⁻¹, respectively.

381 *3.2- Monitoring of the physico-chemical evolution in the liquid phase during biotic* 382 *experiment.*

383 Throughout the experiment, liquid and gas samples were collected in order to perform the
384 different analyses. The solid phase was analyzed before and after the experiment. The liquid
385 phase was composed of the formation water with indigenous microorganisms. As expected
386 from a deep aquifer formation water (- 582 m), the latter is highly reduced with an Eh around
387 -200 mV (Table 1). The ionic water content is dominated by bicarbonate and sulfate amongst
388 anions and calcium amongst cations. The total concentration of dissolved elements in the
389 water can be estimated by summing bicarbonate, sulfate, chloride, calcium, magnesium,
390 potassium and sodium knowing that 4.79 mM (286,6 mg.L⁻¹) represents only 0.8 % of
391 seawater salinity (Banks et al., 2014). Such low salinity strongly suggests that the formation
392 water is of meteoric origin. The concentrations of dissolved Ca⁺ and HCO₃⁻ confirm an
393 interaction between water and carbonate minerals such as lithoclasts observed in the reservoir
394 rock. The origin of sulfate can be hardly depicted and could have resulted from the dissolution
395 of trace amounts of gypsum or anhydrite or from oxidation of sulfides in more crucial
396 conditions. Despite its relatively low concentration, the dominance of sulfate as a terminal

397 electron acceptor in the formation water suggests that sulfate-reducing bacteria are the
398 dominant microorganisms in such ecosystem.

399 One month before its injection into the reactor, the formation water (downhole sample)
400 containing the microorganisms, stored until now at 4°C, was supplemented with wellhead
401 water, sterilized by 0.1µm-filtration and maintained at room temperature. At the beginning of
402 the biotic experiment, sulfate concentration was $9.20 \cdot 10^{-2} \pm 4.60 \cdot 10^{-3}$ mM (*i.e.* $1.28 \cdot 10^{-1} \pm$
403 $6.40 \cdot 10^{-3}$ mmol; Figure 3a). During the experiment, analytical measurements showed an
404 initial decrease in the amount of sulfate after 10 days from the start, followed by its complete
405 consumption on day 32. In order to maintain the dynamic equilibrium, formation water
406 supplemented with sulfate was added. The whole added formation water was filter-sterilized
407 with a 0.1 µm filter to avoid any microbial interference. Three successive water injections
408 were performed on days 48, 104 and 126, respectively. After each injection, an expected
409 increase in sulfate quantity was noted. Sulfate quantity increased by $9.62 \cdot 10^{-2} \pm 4.81 \cdot 10^{-3}$
410 mmol, $5.10 \cdot 10^{-2} \pm 2.55 \cdot 10^{-3}$ mmol and $1.50 \cdot 10^{-2} \pm 7.50 \cdot 10^{-4}$ mmol on days 50, 105 and 131,
411 respectively. Following these increases and before O₂ injection, sulfate consumption resumed
412 and continued until sulfate's complete depletion from the system. During the first 130 days,
413 $2.61 \cdot 10^{-1} \pm 1.31 \cdot 10^{-2}$ mmol of sulfate were consumed. The third injection was applied to refill
414 the sulfate quantity in order to replenish the system stores before O₂ injection.

415 During this first period of the experiment, sulfate quantity showed a notable progressive
416 reduction. However, after oxygen injection into the system, the previously noted progressive
417 reduction in sulfate quantity was noted to subside and even reverse into a remarkable upsurge.
418 This estimated increase in sulfate quantity was of $4.30 \cdot 10^{-2} \pm 2.15 \cdot 10^{-3}$ mmol. Using ionic
419 chromatography coupled with a variable wavelength detector, no sulfide ions were detected in
420 the liquid phase. Similarly no hydrogen sulfide was detected in the gas phase by gas
421 chromatography (1 ppm as minimum detection limit). Taking into account the amount of

422 sulfate depleted from the system, sulfate-reducers were expected to produce $2.61 \cdot 10^{-1} \pm$
423 $1.31 \cdot 10^{-2}$ mmol of sulfide, bisulfide or hydrogen sulfide at most. Even if the sulfur was
424 supposedly only in the form of hydrogen sulfide, it constituted less than 0.006 % of the total
425 gas quantity. The value is too low to be detected by gas chromatography.

426 At the beginning of the experiment and simultaneously with sulfate consumption, an increase
427 in acetate quantity was noted and is shown in Figure 3b. Acetate was produced in the reactor
428 during the first 45 days despite not being detected initially in the formation water. Its quantity
429 increased from zero to $1.23 \pm 6 \cdot 10^{-2}$ mmol at day 13, then attained $2.09 \pm 1 \cdot 10^{-1}$ mmol at day
430 50 with an additional increase until reaching a peak of $2.34 \pm 1 \cdot 10^{-1}$ mmol. This quantity
431 remained stable until the end of the incubation.

432 Similarly to *in situ* conditions, mono-aromatic hydrocarbons including toluene and benzene
433 were supplemented in the gas phase. Monitoring of these compounds was carried out in the
434 liquid phase. At day 8, the molar quantity of toluene was around $2.55 \cdot 10^{-3} \pm 1 \cdot 10^{-4}$ mmol
435 (Figure3c), which decreased after 35 days before increasing again after toluene injection.
436 After this injection, $2.08 \cdot 10^{-3} \pm 1 \cdot 10^{-4}$ mmol were present in the system at day 49. Afterwards,
437 the toluene gradually decreased to $4.12 \cdot 10^{-4} \pm 6 \cdot 10^{-5}$ mmol at the end of the experiment (day
438 179). During 180 days of incubation including, 130 days in anaerobiosis and 50 days in
439 aerobiosis, no benzene degradation was observed in these conditions.

440

441 *3.3- Monitoring of the physico-chemical evolution in the gas phase during biotic experiment.*

442 Methane is the most abundant component in the gas phase. At the beginning of the
443 experiment, 60.4 ± 0.6 bar of the gas mixture $\text{CH}_4 + 1\% \text{CO}_2$ was added to the system at a
444 temperature of 36 °C. Considering the compressibility factor of 0.9 which is calculated by the
445 PhreeqC model based on Peng-Robinson's equation, $4.52 \pm 2 \cdot 10^{-1}$ mol of CH_4 were injected.

446 At day 4, $5.99 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$ mol of CH_4 were lost from the gas phase (Figure 3d). Gas
447 quantities were calculated based on the total pressure measured by the barometer and the gas
448 phase composition analyzed by the gas chromatography. The calculation performed *via*
449 Phreeqc indicated a solubility of $6.25 \cdot 10^{-2}$ mol.Kgw⁻¹ in this experiment's conditions. Thus,
450 taking into account the uncertainty, the CH_4 quantity lost from the gas phase corresponded
451 entirely to its soluble equivalent in the liquid phase. In order to readjust the total pressure after
452 stabilizing the piston position, a second and last injection of the gas mixture was performed
453 on day 6. This injection induced a $2.16 \cdot 10^{-1} \pm 1 \cdot 10^{-2}$ mol increase in methane quantity. After
454 each water injection, a certain number of methane moles transfer to the liquid phase in order
455 to preserve the gas-unsaturated liquid equilibrium. The quantities transferred to the formation
456 water were $6.27 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$ mol, $7.77 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$ mol and $7.13 \cdot 10^{-3} \pm 3 \cdot 10^{-4}$ mol after the
457 first, second and third water injections, respectively. Afterwards, no variations in methane
458 quantity were observed until the end of the experiment. The curve in the Figure 3d represents
459 the variations in methane quantity throughout the experiment based on the thermodynamic
460 model. This model fits well the experimental data obtained during the study.

461 Carbon dioxide was present in an initial proportion of 1 % of the gas mixture, corresponding
462 to an initial quantity of $4.56 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$ mol in the gas phase. Figure 3e showed the
463 variations of CO_2 quantity during the experiment. Similarly, to methane, an initial decrease in
464 CO_2 quantity was observed due to the gas-liquid equilibrium. Following this phase, the
465 CH_4/CO_2 injection applied to readjust the pressure generated an increase in CO_2 quantity of
466 $2.34 \cdot 10^{-4} \pm 1 \cdot 10^{-5}$ mol. Throughout the experiment, a decrease in CO_2 quantity was observed.
467 The last measurement that was done on day 178 showed $1.56 \cdot 10^{-2} \pm 7 \cdot 10^{-4}$ mol of CO_2 in the
468 gas phase. The curve representing the thermodynamic variations of CO_2 in the gas phase was
469 presented in the same figure. The model showed that the thermodynamic equilibrium and
470 gases solubility were reached before the reinjection of CH_4/CO_2 . The reinjection did not have

471 any significant impact on the thermodynamic equilibrium in view of the minimal quantity that
472 was added. The calculated pH was estimated at 5.1. However, the continuous decrease in the
473 carbon dioxide quantity as well as the remarkable difference between the model and the
474 experimental measurements cannot solely be explained by thermodynamic calculations and
475 suggested the presence of an autotrophic metabolism and the incorporation of the carbon
476 inside the biomass.

477 On day 130 of incubation, O₂ was injected in the reactor. At day 131, $4.16 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$ mol
478 were quantified, constituting 1% of the gas phase (Figure 3e). The quantity of oxygen is
479 calculated based on the total pressure of the reactor with a compressibility factor of 0.9 and
480 the molar fraction of O₂ analyzed by the gas chromatography. At the end of the experiment
481 (day 178), $3.57 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$ mol of O₂ remained in the gas mixture. Noting the increase in
482 sulfate in the liquid phase after O₂ injection, 8.60×10^{-2} mmol of O₂ had potentially oxidized
483 sulfide into sulfate. Calculated variations of O₂, taking into account the number of moles lost
484 due to analysis, are represented in the curve. Note that the difference between the
485 experimental and calculated values could be due to potential mineral oxidation that occurred
486 in the reactor such as sulfur oxidation.

487 As previously explained, the experiment aims at studying the effects of the
488 CH₄ -1% CO₂ -1% O₂ mixture in a deep storage aquifer. Hydrogen was not present in the
489 injected gas mixture. However, limited quantities of H₂ near detection threshold were detected
490 between day 4 and day 45 of the experiment as shown in Figure 3f. Before its depletion, the
491 maximum measured quantity at day 31 was estimated at $2.51 \cdot 10^{-4} \pm 1 \cdot 10^{-5}$ mol, which
492 represented 0.005% of the gas phase. In fact, this amount is too low to be considered at that
493 time of the experiment. However, it is worth mentioning that the peak of H₂ was observed at a
494 period when sulfate was undetected in the system. Surprisingly, H₂ accumulation clearly
495 occurred after O₂ injection into the system. The amount of H₂ over time was shown through a

496 positive slope that started one day after oxygen injection and continued until the end of the
497 experiment. The last measurement of H₂ in the gas phase was $1.99 \cdot 10^{-2} \pm 9 \cdot 10^{-4}$ mol, which
498 represented 0.5% of the gas phase. The experimental H₂ values indicated continuous
499 production of this gas. Considering a solubility of $2 \cdot 10^{-4}$ mol.Kgw⁻¹ (via PhreeqC), $1.75 \cdot 10^{-4}$
500 mol of H₂ were present in the liquid phase at the end of the experiment.

501 3.4- Evolution of the bacterial community during the experiment.

502 Besides the importance of identifying O₂ effect on microbial life, analyzing the qualitative
503 and quantitative variations in microbial diversity throughout the experiment can help
504 understanding the changes in the liquid and the gas phases. The presence of archaea could not
505 be demonstrated by PCR and nested-PCR approaches targeting this taxonomic group. The
506 bacterial diversity results based on the sequencing of bacterial 16S rRNA genes were
507 combined with the results of quantification of these same genes obtained by quantitative PCR
508 in each sample (Figure 4). At the beginning of the experiment, the microbial community of
509 $2.1 \cdot 10^5 \pm 1.3 \cdot 10^5$ cells/mL stored since five months at ambient pressure at 4 °C was
510 dominated by *Geobacteraceae* > *Desulfovibrionaceae* > *Rhodocyclaceae* > *Spirochaetaceae*.
511 The proportions of each of the aforementioned strain species were noted to vary during the
512 first 43 days of incubation. On one hand a reduction in *Geobacteraceae* and
513 *Desulfovibrionaceae* was observed, in contrast to a growth in *Spirochaetaceae* and
514 *Peptococcaceae*. This new equilibrium in the community was relatively stable until
515 incubation day 105 when the *Peptococcaceae* concentration was noted to be increasing.
516 *Peptococcaceae* growth coincided with the reduction in toluene in the liquid phase (Figure
517 3c). The oxygen injection had a significant impact on the microbial community presented by a
518 substantial reduction in the bacterial cell concentration from $1.1 \cdot 10^5 \pm 2.0 \cdot 10^4$ cells/mL (t₁₃₀)
519 to $3.7 \cdot 10^3 \pm 1.2 \cdot 10^3$ cell/mL (t₁₃₃) reaching an endpoint of $4.1 \cdot 10^2 \pm 8.4 \cdot 10^1$ cell/mL (t₁₇₉).
520 *Desulfovibrionaceae* and *Sphingomonodaceae* retained a detectable presence despite having

521 higher concentrations prior to oxygen injection. Members of the *Sphingomonodaceae* may
522 have aerobic metabolism in addition to having fermentation capacities allowing them to
523 produce H₂ by degrading organic molecules.

524 Via the 16S rRNA gene, the bacterial taxonomic diversity of active cells was monitored and
525 the results are presented in the Figure 5 and Table 2. The bacterial strains' activity seems to
526 be restricted and different from that seen at atmospheric pressure (intensity of activity at day 0
527 compared to the activity during the incubation). Thus, the *Geobacteraceae* (blue),
528 *Rhizobiaceae* (brown), *Desulfovibrionaceae* (black yellow, below) and *Spirochaetaceae* (dark
529 blue) were the most active in the original community obtained from the sampler that evolved
530 for several months at atmospheric pressure in anaerobiosis. Just before O₂ injection (day 130),
531 strong activities of *Desulfovibrionaceae* (yellow), *Peptococcaceae* (green) and
532 *Thermodesulfovibrionaceae* (blue) were detected. On day 179 at the end of the incubation and
533 even though their microbial activity were quite low, *Spirochaetaceae* and
534 *Desulfovibrionaceae* strains could still be detected. Hence, the sulfate-reducing metabolic
535 group had suffered but has not completely disappeared.

536 In addition, the liquid phase was sampled prior to O₂ injection in order to target and cultivate
537 sulfate-reducers and fermenters in special media, as those microorganisms are considered key
538 players in deep aquifers. From the sulfate-reducers' medium, the strain 15A, a representative
539 of the *Desulfovibrio vulgaris* strains, was isolated and identified (99.72% identity with 16S
540 rDNA sequence of *D. vulgaris* strain "Miyazaki F"). The strain 15A had a strictly anaerobic
541 growth. It was capable of growth with CO₂ and H₂ as sole carbon and energy sources.
542 Regarding its heterotrophic growth, formate oxidization was tested and turned to be
543 incomplete with noted production of acetate. As expected, the strain 15A did not produce
544 acetate when grown autotrophically. Two fermenters were isolated, strain 2A and strain
545 Recti_s6. Strain 2A is phylogenetically close to *Pleomorphomonas koreensis* (99.70% of

546 identity; 16S rDNA). Grown on glucose-based medium, strain 2A produces formate, acetate,
547 CO₂ and H₂ even in oxic conditions such as the air in the head space. Strain Recti_s6 is
548 related to *Rectinema cohabitans* strain HM (99.83% of identity) which a spirochaete isolated
549 from an anaerobic naphthalene-degrading enrichment culture (Koelschbach et al., 2017).
550 Finally, representatives of the *Bacillus* genus were also detected through the identification of
551 their characteristic structures and spore forms (data not shown). These bacteria are strictly
552 aerobic and are able to maintain under anoxic conditions through their ability to sporulate.
553 Spores protect these bacterial until more favorable conditions for germination occur.

554 3.5- Evolution of the solid phase during the experiment.

555 XRD was performed on the reservoir rock that is sampled from the deep aquifer and
556 introduced in the reactor and on the solid sample after the experiment. At the end of the
557 experiment, X-ray diffraction performed on the collected rock did not demonstrate any
558 significant changes in the solid crystallized phases before and after the experiment (Figure 6).
559 Mainly composed of quartz with minor calcite components, the solid sample did not vary
560 significantly after the experiment. However parallel measurements were performed on a
561 reservoir rock sample that was immersed in the formation water containing the microbial life
562 with the substitution of CH₄-CO₂-O₂ for N₂ gas. These measurements indicated the formation
563 of iron sulfide (Figure 6). Thus the possibility of iron sulfides production during the
564 experiment with its subsequent oxidation after O₂ injection cannot be excluded. SEM-EDX
565 showed high amount of clay minerals in between quartz grains, and Fe and S colocalization
566 suggested the presence of iron sulfides in agreement with XRD (Figure 7). No variations was
567 observed by depth in the basket and no biofilm could be detected in these conditions.

568 The average porosity obtained by means of X-ray tomography from four different capillaries
569 did not show any significant variation with an initial porosity of around 43.5 % and a final
570 one of around 44.5% with an estimated uncertainty of ± 2 %. Thus, no recrystallization or

571 potential biofilm could be demonstrated in agreement with mineralogical observations. Note
572 that black particles were observed in the solid phase at the end of the experiment. Similar
573 black particles produced during a bio-geochemical interactions study were identified as pyrite.

574

575 **4. Discussion**

576 The role and contribution of green and renewable energy in the energy mix is more than ever
577 required in order to counteract and reduce the notorious effects of greenhouse gas emissions.
578 As part of energy transition, natural gas will become greener through the production and
579 storage of biomethane. However, despite that biomethane is an attractive source of renewable
580 energy, the effects of its storage particularly in massive amounts remains unknown. Thus,
581 identification and comprehension of associated phenomena to biomethane storage is
582 fundamental for the basis of any territory's energy strategy. In order to secure energy
583 supplies, to face consumption peaks and variations between summer and winter, a significant
584 part of natural gas can be stored in geological structures such as salt cavities or deep aquifers.
585 That said, biomethane could replace natural gas on the long term and can be stored within
586 these geological structures. Unlike natural gas, O₂ is added to biomethane during sulfide
587 reoxidation process before injection into the gas network. To our knowledge, this is the first
588 study to evaluate the potential impact of O₂ in anoxic ecosystems such as deep aquifers in this
589 setting. The scenario tested in this study is the most unfavorable case of biomethane storage
590 consisting of an O₂ supply of 1% in the context of an Eocene aquifer which is mainly
591 composed of sands, sandstones and carbonate deposits (Brenot et al., 2015). Based on the X-
592 ray diffraction results, calcite, iron sulfides (pyrite and marcasite), oxi-hydroxides (maghemite
593 and hematite) and clay minerals were detected in traces. The monitoring well Ab_L_1
594 sampled for this study is the closest to the natural gas bubble (Ranchou-Peyruse et al., 2019).
595 At an approximate depth of 600 m, interactions between the formation water and the injected

596 gas are at their peak. Under a high pressure of 60 bar, oxygen solubility is more important
597 than its solubility at the surface even at a 1% proportion. The Ab_L_1 formation water has a
598 very low salinity of 4.79 mM (286.56 mg.L⁻¹) which is equivalent to only 0.8 % of marine
599 salinity (Table 1). However the abiotic experiment showed salt release from the solid into the
600 liquid phase despite rinsing of the rock during the preparation. Calcium and chloride were the
601 main ions detected. Calcium chloride is a brine known to be used during drilling operations.
602 Nevertheless, stoichiometry suggests another origin for calcium which could be released from
603 the calcite detected in the reservoir rock. Even in small quantities of few mmoles, changes in
604 the liquid's salinity have the potential to alter the thermodynamic equilibrium.

605 4.1- Sampling and pre-incubation.

606 The formation water containing indigenous microorganisms was sampled 5 months before the
607 start of the high-pressure biotic experiment. Water was collected using a downhole sampler
608 ensuring the purity of the microbiological sample and maintaining it under high-pressure until
609 reaching the laboratory. Under controlled conditions, the pressure was gradually lowered in
610 order to avoid damaging or destroying the microorganisms through rapid depressurization.
611 The formation water was then stored at ambient temperature and under anoxic conditions with
612 N₂ as gas phase. One month before the experiment, the formation water with the
613 microorganisms was supplemented with sterile water from the same study site in order to
614 reactivate the microorganisms. During this pre-incubation period, the microorganisms already
615 started to consume different elements present in the water particularly sulfate. Once injected
616 in the reactor, liquid phase was supplemented with sulfate by the release of these ions from
617 the rock. Sulfate ions present in the solid phase may be due to pyrite oxidation or to drilling
618 brines. Incubation at atmospheric pressure seems to have particularly favored the
619 *Geobacteraceae* family, which proportion drastically decreased in the pressurized reactor.
620 Similar changes affected the *Desulfovibrionaceae* family which are sulfate-reducing

621 proteobacteria previously described in this site (Ranchou-Peyruse et al., 2019). These findings
622 encourage undergoing further studies assessing the behavior of microbial communities in
623 deep environments under high pressures, even in continental undergrounds where pressures
624 are known to be lower than deep marine ecosystems.

625 *4.2- Physicochemical and bacterial evolution before oxygen injection.*

626 The objective of the custom-designed reactor in this study is to simulate as precisely as
627 possible a gas storage aquifer. Anoxic conditions were applied from the beginning of the
628 experiment with a gas phase composed of CH₄ with 1% of CO₂ and small quantities of
629 benzene and toluene. The entire rock was initially immersed in the formation water during the
630 first 5 days in order to colonize it with microorganisms. Afterwards the water level was
631 lowered allowing the upper part of the rock to bath in the gas phase whereas its lower part
632 remained in the liquid phase with the microorganisms. Microbial communities were able to
633 grow under a pressure of 60 bar and a temperature of 36 °C, similarly to the aquifer's *in situ*
634 conditions. The thermodynamic modeling studies helped to identify the equilibrium status of
635 our system. The parameters such as the gas quantity and volume, the aqueous phase
636 composition and quantity and the temperature of the system were fixed. PhreeqC model
637 calculated the solubility of each gas in the aqueous phase under the system's conditions, and
638 the quantity of each gas in the gas phase at equilibrium. At day 5 and before the reinjection of
639 CH₄+CO₂, the quantities of CH₄ ($4.46 \pm 2.2 \cdot 10^{-1}$ mol) and CO₂ ($2.97 \cdot 10^{-2} \pm 1.5 \cdot 10^{-3}$ mol) in
640 the gas phase were equal to those calculated by PhreeqC (4.41 and $3.37 \cdot 10^{-2}$ mol),
641 respectively. These findings confirm that the thermodynamic equilibrium was attained on day
642 5. The re-injection of the gas mixture on day 6 inducing a small increase in the quantities of
643 these gases (Figures 2d and 2e) did not cause a significant difference in the thermodynamic
644 equilibrium. On day 20, the reduction in CO₂ quantity from the gas phase was greater than the
645 value calculated at the thermodynamic equilibrium suggesting an additional consumption

646 which can only be explained by integration of CO₂ in the bacterial biomass. The detection of
647 H₂ followed by its consumption supports this hypothesis. Carbon dioxide and hydrogen are
648 consumed by autotrophic microorganisms that use H₂ as a source of energy and electrons. The
649 absence of archaea in the microbial community confirms the lack of CO₂/H₂ consumption *via*
650 methanogenesis in this study. Since the beginning of the experiment, sulfate reduction was
651 noted in the liquid phase in the absence of O₂. That observation necessitated three sulfate-
652 supplemented formation water injections in order to maintain the incubation. The bacterial
653 community analysis confirmed the presence of the *Desulfovibrionaceae* family (Figure 4).
654 Moreover, the 16S rRNA transcripts studies confirmed their activity in the original
655 community (Figure 5). Previous studies on the Ab_L_1 site recognized the activity of the
656 sulfate-reducing bacteria, particularly the delta-proteobacteria which include the
657 *Desulfovibrionaceae* family (Ranchou-Peyruse et al., 2019). We conclude that the anaerobic
658 activity of sulfate-reducing bacteria was responsible for the depletion of sulfate from the
659 system. The *Desulfovibrio vulgaris* 15A strain isolated in this study showed an ability to
660 produce acetate under heterotrophic conditions. Acetate level was noted to increase during the
661 first month of incubation and remained stable until the end of the experiment suggesting a
662 simultaneous absence of acetoclastic bacteria (Figure 3b). Moreover, acetate was not among
663 the ions released from the solid phase during the abiotic experiments. Acetate could have also
664 been produced by fermentative bacteria present in the community such as isolated
665 *Pleomophomonas koreensis* 2A and *Rectinema cohabitans* Recti_s6 strains. Under
666 fermentative conditions, these two strains have been shown to produce formate, acetate, CO₂
667 and H₂. Therefore we conclude that in the first days of incubation, there was a significant
668 activity of fermenters particularly *Spirochaetaceae* and heterotrophic sulfate-reducers. The
669 latter reduce sulfate from organic molecules present in the water, mineral matrix but also in
670 the necromass. It is also evident that the H₂ produced during fermentation was directly

671 consumed by the sulfate-reducers including the *Desulfovibrio vulgaris* 15A strain, as sulfate
672 depletion coincided with H₂ detection. The hydrogenotrophic ability of certain strains of the
673 *Desulfovibrio* genus has been previously reported (Bagnoud et al., 2016; Detmers et al.,
674 2004). Conversely, sulfate supplementation resulted in H₂ disappearance. Similarly, the
675 reduction in CO₂ (Figure 3e) could be partially explained by this autotrophic growth that uses
676 this carbon source for its biomass and metabolism. However the sulfide compounds produced
677 through the sulfate-reduction metabolism were not detected in the liquid nor the gas phases.
678 The black particles observed in the solid basket and the reactor at the end of the experiment,
679 identified as pyrite by XRD, suggest that iron sulfide was formed by the interaction of ferrous
680 irons present in the reservoir rock and the formation water with the produced sulfide
681 compounds (Berlendis et al., 2010; Caldwell et al., 2008; Fry et al., 1997; Park et al., 2006;
682 Zettlitzer et al., 2010).

683 4.3- Monoaromatic hydrocarbons biodegradation.

684 Several studies have demonstrated the potential for biodegradation of monoaromatic
685 hydrocarbons by autochthonous microbial communities in deep aquifers used for natural gas
686 storage (Berlendis et al., 2010; Aüllo et al., 2016; Ranchou-Peyruse et al., 2017). In this study,
687 the gas phase was supplemented with benzene and toluene at concentrations similar to those
688 typically found in natural gas (7.95 ppm of benzene and 3.57 ppm of toluene). A reduction in
689 toluene started around day 100 until the injection of O₂. No toluene reduction was noted in
690 oxic conditions. A growth of the *Peptococcaceae* strains (Figures 4 and 5) can be noted in the
691 same period. This bacterial family includes gram-positive sulfate-reducing and fermentative
692 bacteria that have previously been associated with biodegradation of monoaromatic
693 hydrocarbons in similar environments (Abu Laban et al., 2010; Aüllo et al., 2016;
694 Kleinsteuber et al., 2008; Liu et al., 2004; Morasch et al., 2004; Ranchou-Peyruse et al., 2017;
695 Robertson et al., 2000; Taubert et al., 2012). This hypothesis is consistent with the current

696 level of understanding of the behavior of microbial communities at the gas bubble / formation
697 water interface. In close proximity to the gas bubble, exogenous molecules specifically carbon
698 molecules transferred from the gas to the formation water become more accessible to the
699 microorganisms. This zone that is less oligotrophic than the rest of the aquifer appears to be
700 more favorable for the development of microorganisms such as *Desulfovibrio*. The
701 *Desulfovibrio* genus is known to be more competitive and faster growing than
702 *Desulfotomaculum* (*Peptococcaceae*) when present in environments with abundant organic
703 molecules (Aüillo et al., 2013). After O₂ injection, a notable decline in sulfate reduction,
704 microbial activity and toluene degradation were noted. Terminating the natural bio-
705 attenuation of aromatic hydrocarbons could be a side effect of massive O₂ influx into UGS
706 and should not be neglected in the management of these geological storages.

707 *4.4- Evolution of the four-phase system after oxygen injection.*

708 Once the O₂ was injected in the gas phase and based on abiotic physicochemical reactions, the
709 simulation model predict that the value at saturation ($4.42 \cdot 10^{-4}$ mol.Kgw⁻¹) in the liquid phase
710 was experimentally reached 5 days after the O₂ injection (*i.e.*, t₁₃₆). However, the
711 experimental data showed a constant decrease in the gas phase of O₂ until the end of the
712 experiment. This reduction is linked to: i) the solubilization of O₂ in the liquid phase (0.67
713 mmol estimated from the model); ii) the reoxidation of sulfide into sulfate (8.60×10^{-2} mmol),
714 the sulfide potentially trapped in the form of iron sulfides in the rock matrix (*e.g.* pyrite).
715 Many studies showed the immediate underground oxidation of pyrite after O₂ injection in the
716 storage systems (Guo et al., 2021; Kushnir et al., 2012; Lu et al., 2016; Vu et al., 2018)
717 causing an increase in sulfate quantities (Yuan et al., 2009); iii) and a possible reoxidation of
718 other reduced elements, such as ferrous ions. The decrease in O₂ quantity was more
719 significant and may be due to different gas-solid interactions not identified during our study.
720 The solubility of O₂ simulated by the model can be estimated to be around $4.42 \cdot 10^{-4}$

721 mol.Kgw⁻¹ in the liquid phase during the last 50 days of the experiment. If a comparable water
722 is placed under different environment and conditions such as air and 36 °C temperature, O₂
723 saturation would be of around 2.1·10⁻⁴ mol.Kgw⁻¹ at equilibrium (Benson and Krause, 1980;
724 Millero et al., 2002) and an aerobic microbial ecosystem would develop (Stiff et al., 1992).
725 The hyperoxic water in our reactor was clearly toxic to the strictly anaerobic microorganisms,
726 as evidenced by the drastic decrease in bacterial taxonomic diversity and its activity from the
727 day following O₂ injection (Figures 4 and 5) even if the existence of some anoxic micro-
728 niches could be supposed. It is probable that part of O₂ could have reacted with the organic
729 matter resulting from necromass.

730 Some microorganisms survived these new environmental conditions and retained a metabolic
731 activity as demonstrated by a surprising constant production of H₂ following O₂ injection that
732 persists till the end of the experiment. Analyses performed on fermentative strains 2A and
733 Recti_s6 demonstrated their capability to produce H₂ in oxic conditions. With the reduction in
734 the majority of strict anaerobic bacteria and the decline in hydrogenotrophic sulfate-reducers
735 such as *Desulfovibrio vulgaris* 15A, sulfate and H₂ were no longer consumed, explaining its
736 accumulation in the gas phase. Despite that some strains of the *Desulfovibrionaceae* family
737 were able to survive O₂, their activity is significantly slow and even negligible. A study
738 conducted by Yuan et al. showed the occurrence of sulfate-reduction in anaerobic
739 environment after undergoing phases of oxygen stress. Sulfate-reducing bacteria can have a
740 relative temporary resistance to oxic environments (Yuan et al., 2009) which explains the
741 detection of their low activity. It is possible that some aerobic microorganisms such as
742 *Bacillus* strains and/or aero-anaerobic microorganisms such as *Sphingomonadaceae* may have
743 consumed some of the O₂ as well (Kosako et al., 2000).

744 Despite that the solid phase demonstrated no changes in porosity % or mineral composition
745 among others, the pre and post experimental analyses cannot completely rule out the

746 possibility of potential changes secondary to O₂ injection in the UGS. In fact, the reservoir
747 rock sample was in contact with O₂ present in the air before the experiment and was scanned
748 by X-ray tomography and XRD in oxic conditions. During the experiment, interactions
749 occurred in anoxic environment and produce minerals such as metal sulfides. Following O₂
750 injection, changes such as mineral oxidation occurred. At this stage, the solid phase was in
751 oxic conditions, similarly to its state before the beginning of the experiment. There were no
752 signs suggesting that the metal sulfides precipitates could have significantly reduced the
753 porosity of the rock prior to oxygen injection. However considering these facts, further studies
754 are required in order to assess the state of the solid phase after incubation and before O₂
755 injection in the system.

756 During the multidisciplinary experiment, oxygen effects on the different phases of an aquifer
757 during biomethane storage were assessed. Oxygen injection induced mineral oxidation to the
758 reservoir rock (Lu et al., 2016; Vu et al., 2018), especially iron sulfide oxidation causing
759 increase in sulfate concentration in the aqueous phase. In fact, iron sulfide oxidation was
760 already observed during CAES (Guo et al., 2021; Jung et al., 2013) and CCS (Jung et al.,
761 2013; Shao et al., 2014; Wei et al., 2015) where oxygen was injected as impurity. On a larger
762 scale, reaction of minerals and their dissolution in the aqueous phase can alter the reservoir
763 rock's porosity and therefore affect the storage volume. Another clear result obtained was the
764 deleterious effect of oxygen on the anaerobic microbial communities. Many studies were
765 conducted to confirm the capability of these communities to underground biodegrade
766 hydrocarbons, especially the sulfate-reducing bacteria (Aüllo et al., 2013; Ranchou-Peyruse et
767 al., 2019). Even though the microbial concentration drastically decreased after O₂ injection,
768 some of the communities were able to survive. In order to develop, those communities might
769 adapt to the new aquifer conditions. To get their needs of carbon, microorganisms might
770 develop capabilities for hydrocarbon biodegradation and therefore, the microbial community

771 would adapt to their new environment. During CCS or biomethane storage in deep aquifers,
772 oxygen's effects should be considered on many levels depending on the storage conditions
773 such as the reservoir rock's composition and the microbial communities harbored *in-situ*.

774 **5. Conclusion**

775 In this experiment, we were able to simulate the aquifer Ab_L_1 situated in South Aquitaine,
776 Southwest France in a reactor. Through a multidisciplinary approach, the impact of 1% of O₂
777 under 60 bar total pressure was evaluated in a UGS aquifer for the first time ever. The
778 relatively simple composition of the rock matrix at the Ab_L_1 site mainly containing sand
779 and to a lesser degree calcite did not seem to react after the addition of O₂ at low
780 concentration. Reoxidation of metal sulfide precipitates was expected with subsequent
781 minimal increase in the porosity of the aquifer rock. As of the early 1990s, sulfate was noted
782 to decrease in the Ab_L_1 site as a result of the sulfate-reducing activity amplified by the
783 proximity of the gas bubble and by the consumption of organic molecules dissolved in water
784 by sulfate-reducers (1992: ≈ 18 mg/L SO₄²⁻, 2013: < 7 mg/L SO₄²⁻; (Ranchou-Peyruse et al.,
785 2019)). The reoxidation of metal sulfides would therefore contribute to the restoration of
786 higher sulfate concentrations. Similarly the direct effect on the gases stored in the aquifer
787 (99% CH₄, 1% CO₂) seemed negligible and even absent. Conversely, O₂ had a deleterious
788 effect on the strictly anaerobic microbial community interacting with the UGS. This effect
789 manifested as a decline and even a complete arrest of the natural bioattenuation of
790 monoaromatic hydrocarbons, a decrease in oxygen amount and finally a surprising H₂
791 accumulation in the gas phase deriving from fermentative bacteria able to survive the new
792 oxic conditions.

793 The different biological, geological and chemical impacts of biomethane storage containing
794 1% of O₂ as impurity were evaluated in this study. It will be necessary to carry out new
795 studies with lower oxygen concentrations and for other storage conditions (temperature,

796 pressure, rocks). Nevertheless, in view of these first results, the elimination of oxygen from
797 the gas mixture before its injection must be considered. However oxygen is widely used
798 during different stages of the processing of various other resources such as in CCS or CAES
799 in geological sites. The findings noted in our study shed a new light on O₂ gas effects on the
800 underground environment which is an important step in the green energy era. Such
801 observations lay the cornerstone for the future selection of accessible, secure and stable ways
802 of biomethane underground storage which is crucial for building a successful global clean
803 energy strategy. Further studies involving the use of various amounts of oxygen during
804 storage of different gases compositions and their subsequent implications are required for a
805 better understanding of the static and dynamic interactions occurring during gas storage
806 practices.

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813 **Author Contributions Statement**

814 FC, MRP, PC and ARP co-conceived the study. PH, JM, FC, PC carried out the high pressure
815 and simulation experiments. MG, MRP and ARP applied the microbiological approaches. PS,
816 M-PI, IS, PM and GH used the imaging and mineralogical characterization. ML and ILH
817 analyzed the hydrocarbons. All authors contributed in the interpretation of results and paper
818 writing.

819 **Declaration of interests**

820 DD and PCh are employed by two French companies specialized in geological natural gas
821 storage which are Storengy and Teréga respectively.

822 **Conflict of interest**

823 No conflict of interest declared in relation to the work described.

824

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- 1040

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1042 **Tables and Figures**

1043 **Table 1** - Physico-chemical parameters and compounds of the formation water sampled from
1044 the deep aquifer (Ab_L_1 site), analyzed at atmospheric pressure.

1045 **Table 2**- Standard deviation related to the 16S rRNA transcripts

1046 **Figure 1** - Scheme of the experimental apparatus used during the experiment.

1047 **Figure 2**- Diagram representing the different steps and analyses undergone through the biotic
1048 experiment.

1049 **Figure 3**- Results of the liquid and gas analyses during the biotic high pressure experiment
1050 (60 bar). The vertical line at day 6 represents the CH₄+CO₂ reinjection. The vertical dotted
1051 lines at days 48, 104 and 126 represent sulfate injections. The vertical line at day 132
1052 represents the oxygen injection. a) Sulfate variation in the liquid phase; b) Acetate variation in
1053 the liquid phase; c) Toluene (represented by the blue squares) and benzene (represented by the
1054 red triangles) variations in the liquid phase. The vertical line at day 47 represents toluene
1055 injection. d) Methane variation in the gas phase. The red dots and line represent the calculated
1056 values; e) Carbon dioxide (represented by the green squares) and oxygen (represented by the
1057 red triangles) variations in the gas phase). The lines represent the calculated values; f)
1058 Hydrogen variation in the gas phase.

1059 **Figure 4**- Evolution of the bacterial community during the incubation at 60 bar, 36°C and
1060 with a gas mixture (CH₄, 1%CO₂, 7.95ppm of benzene and 3.57 ppm of toluene) before and
1061 after oxygen injection (1%). The taxonomic diversity is monitored by 16S rRNA genes
1062 sequencing and the quantification is estimated by qPCR of 16S rDNA gene copies for each ml
1063 of sampled water through the experiment. 3a) Monitoring over the 180 days of the
1064 experiment. Following the oxygen injection, the quantity of microorganisms has drastically
1065 decreased. In order to observe the taxonomic diversity, the boxed part is zoomed in figure 3b.

1066 The variation of the sulfate quantity in the liquid phase is represented by the orange dots.

1067 Figure 3b- represents a zoom in on results after O₂ injection.

1068 **Figure 5-** Evolution of the active bacterial community (during the incubation at 60 bar, 36°C

1069 with a gas mixture (CH₄, 1%CO₂, 7.95 ppm of benzene and 3.57 ppm of toluene) before and

1070 after oxygen injection (1%). The taxonomic diversity is based on the sequencing of cDNA of

1071 the 16S RDNA copies and the quantification was estimated by qPCR of cDNA for each mL

1072 of sampled water through the experiment.

1073 **Figure 6-** X-ray diffraction patterns of samples collected before the experiment (T0) and after

1074 the experiment at three depths (top, middle, bottom). I: illite, K: kaolinite, M: maghemite, Q:

1075 quartz, C: calcite, Ms: marcassite, P: pyrite.

1076 **Figure 7-** SEM-EDX observations from surface samples collected at the end of experiment

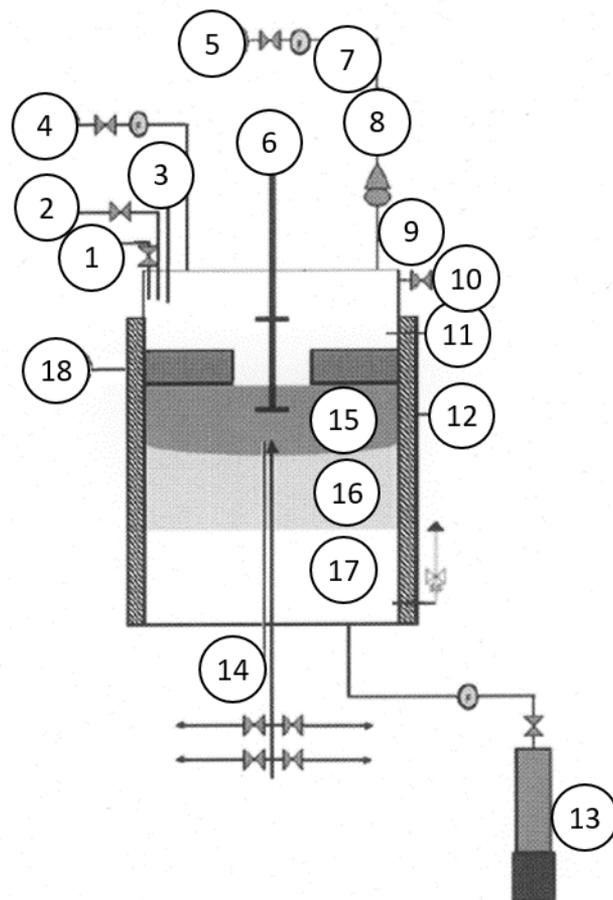
1077 showing clay minerals and quartz (A, SE mode)) and observation of iron sulfides (B, BSE

1078 mode).

1079

The different components:

- 1- Liquid Input
- 2- Gas Output
- 3- Gas temperature probe
- 4- Gas Mixture Input
- 5- O₂ Input
- 6- Stirrer Motor
- 7- Barometer
- 8- Flowmeter
- 9- Barometer
- 10- Gas Output
- 11- O₂ sensor
- 12- Regulation temperature
- 13- Pressure regulation pump
- 14- Liquid Temperature probe
- 15- Liquid Phase
- 16- Piston
- 17- Water from the pressure regulation pump
- 18- Basket containing reservoir rock and capillaries



- Formation water sampling from the well under pressure.
- Liquid phase preparation and sampling for microbial analysis and ionic chromatography.
- Solid treatment, capillary preparation, X-ray tomography, XRD-SEM.

- Ionic and gas chromatography (weekly, before and after each liquid and gas injection).
- Benzene and toluene quantifications (before and after each liquid and gas injection).
- Liquid sampling for microbial analysis (every 10 to 15 days, before and after each liquid and gas injection).
- Oxygen injection at t_{130} .

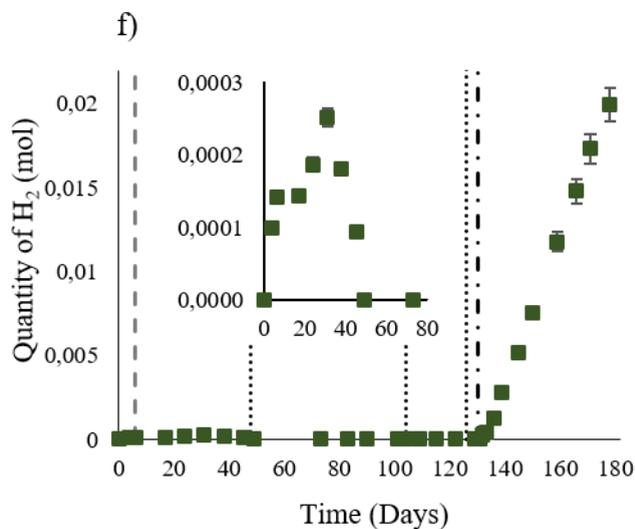
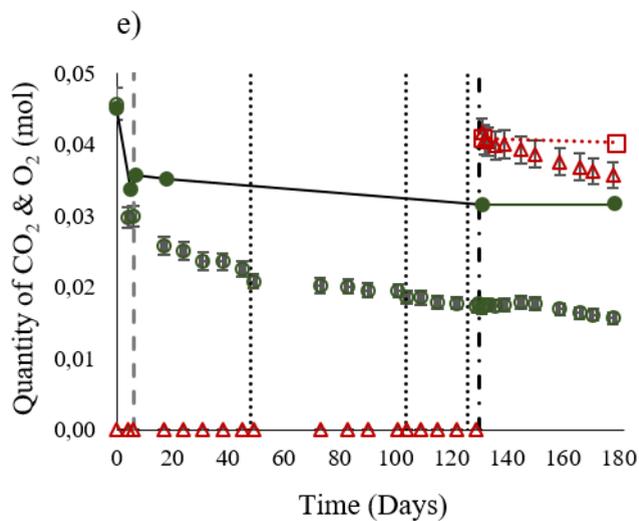
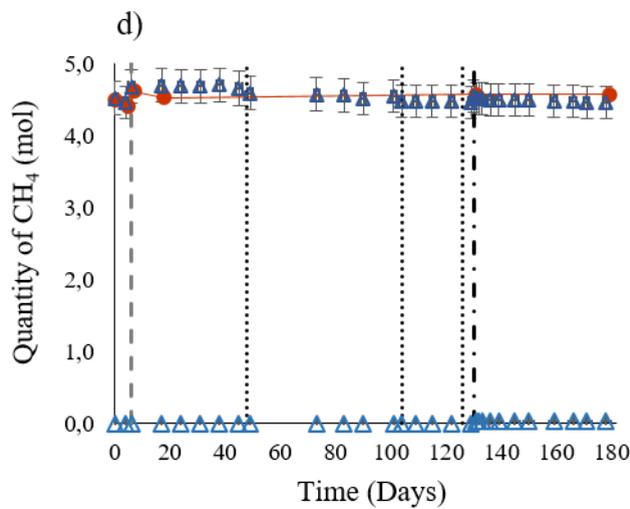
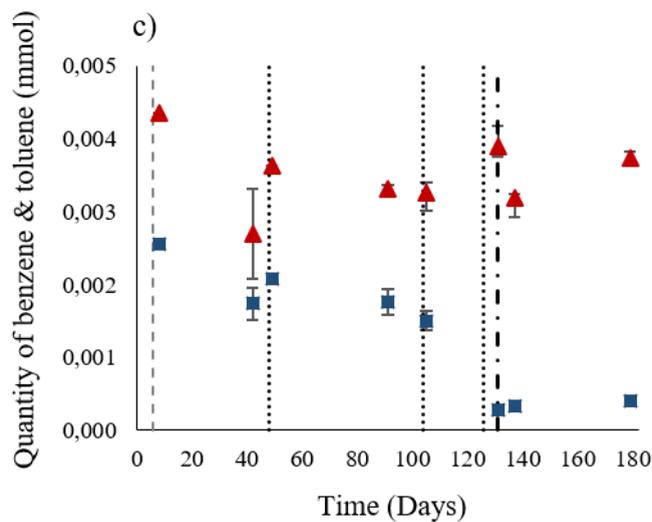
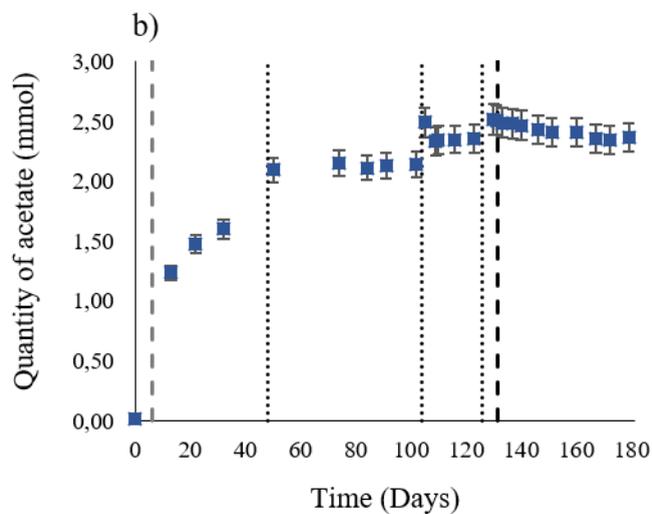
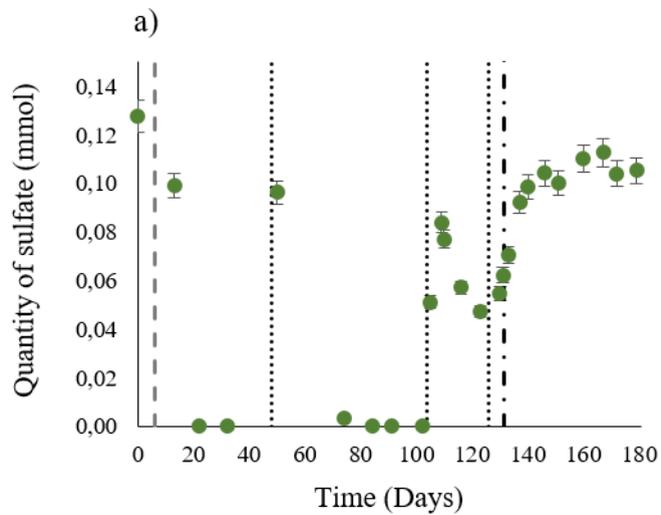
- Liquid sampling for microbial analysis and cell culture
- Solid sampling from the basket for X-ray tomography and XRD-SEM.

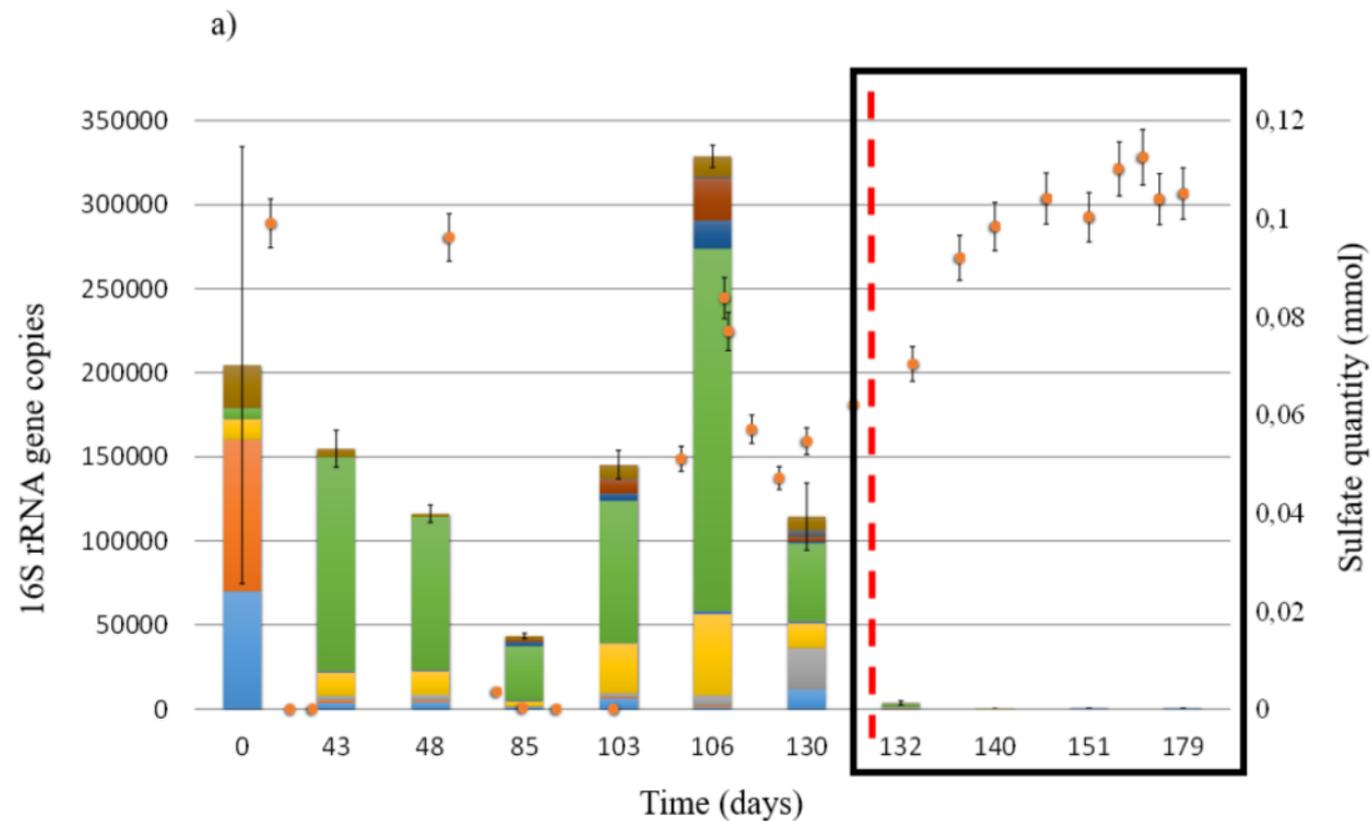
t_0

- Solid basket deposition in the reactor.
- Reactor sterilization.
- Liquid phase injection.
- Gas phase (CH_4 - CO_2 -benzene-toluene injection).

t_{180}

- Liquid phase total sampling.
- Gas phase evacuation.
- Solid basket removal under anaerobic conditions.

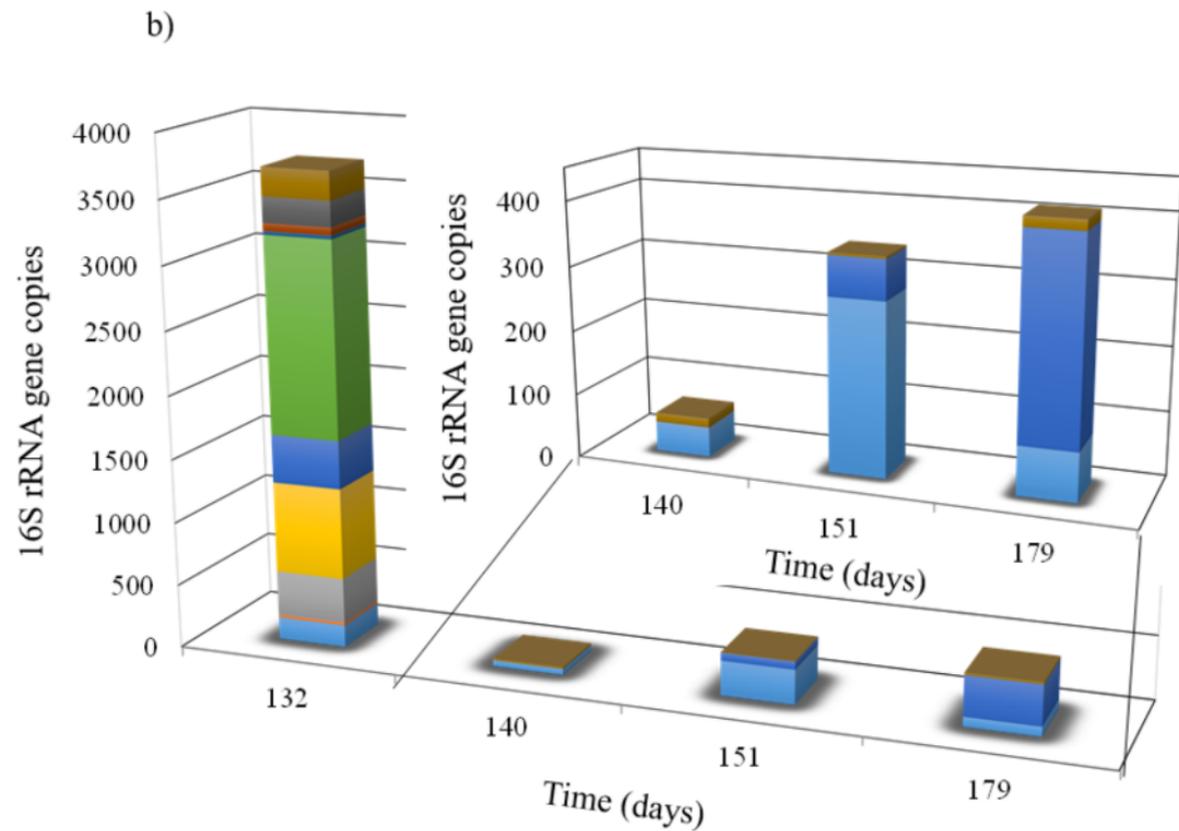




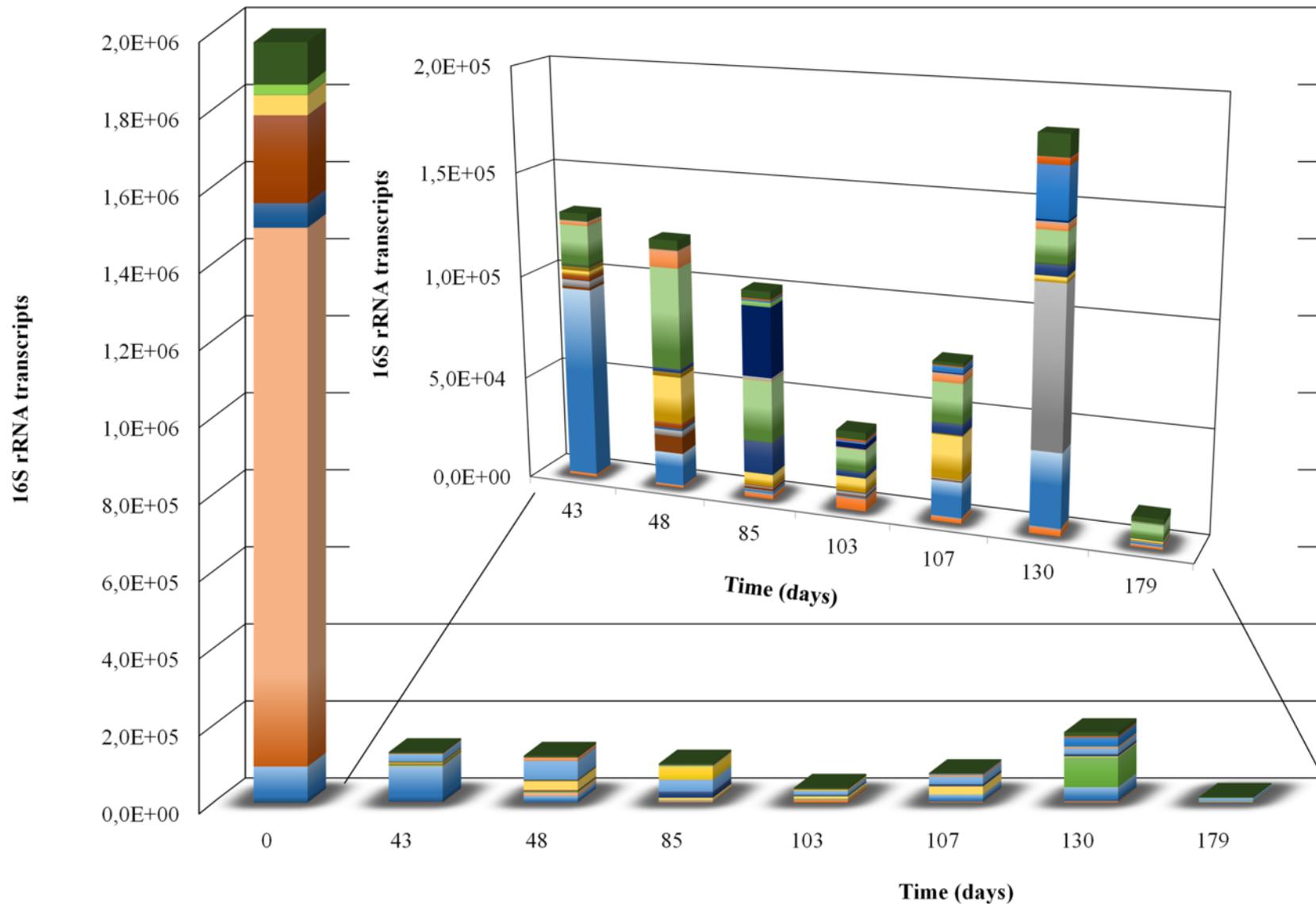
■ *Desulfovibrionaceae*
 ■ *Sphingomonadaceae*
 ■ *Thermodesulfovibrionaceae*

■ *Geobacteraceae*
 ■ *Spirochaetaceae*
 ■ Other

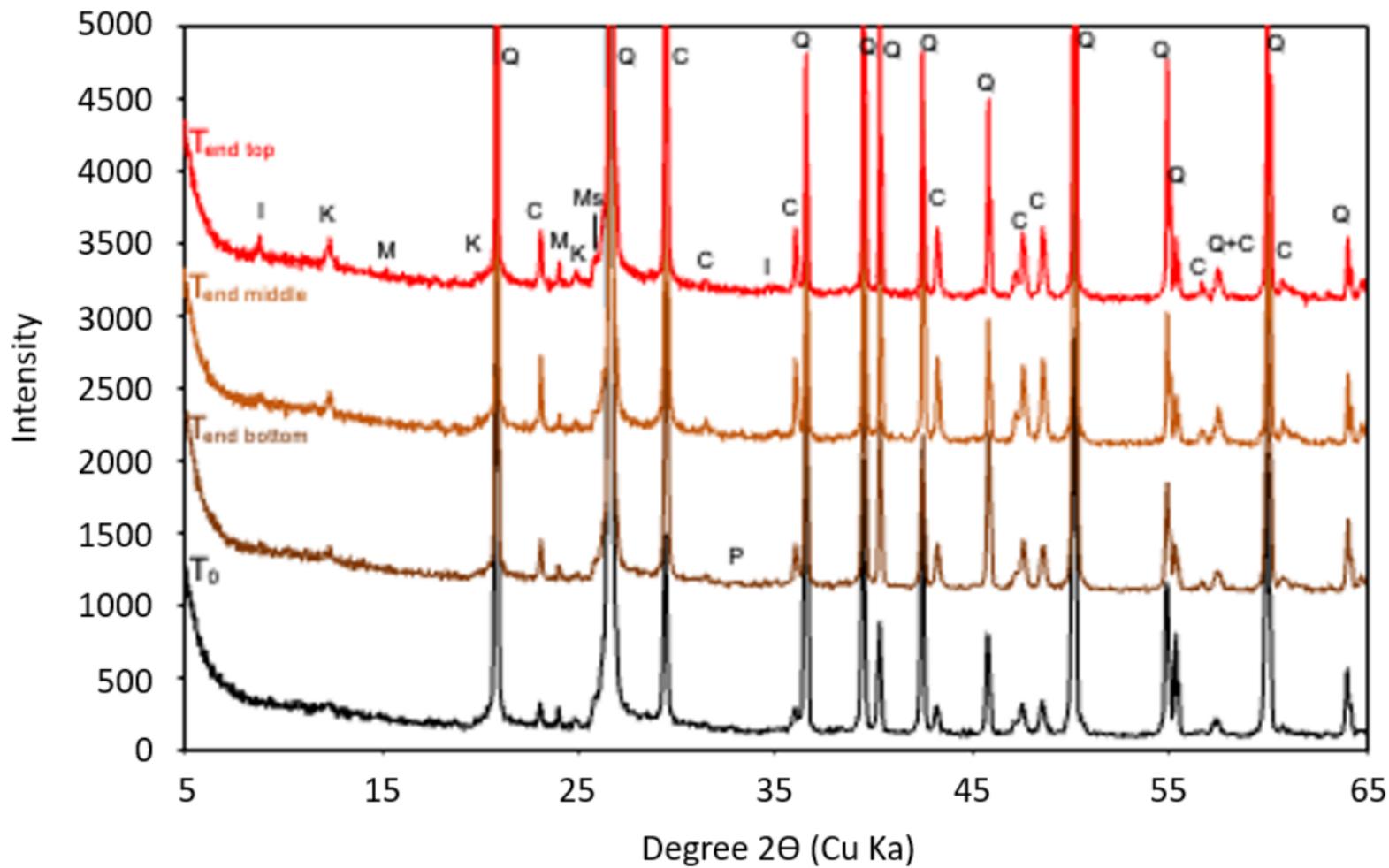
■ *Peptococcaceae*
 ■ *Syntrophaceae*
 ● Sulfates



■ *Rhodocyclaceae*
 ■ *Thermoanaerobacteraceae*



- Other
- Unknown family
- Thermodesulfovibrionaceae
- Thermoanaerobacteraceae
- Syntrophomonadaceae
- Syntrophaceae
- Staphylococcaceae
- SR-FRB-L83
- Spirochaetaceae
- Sphingomonadaceae
- Solibacteraceae (Subgroup3)
- Ruminococcaceae
- Rhodocyclaceae
- Rhizobiaceae
- Pleomorphomonadaceae
- Peptococcaceae
- Geobacteraceae
- Desulfovibrionaceae
- Corynebacteriaceae
- Bukholderiaceae
- Acetobacteraceae



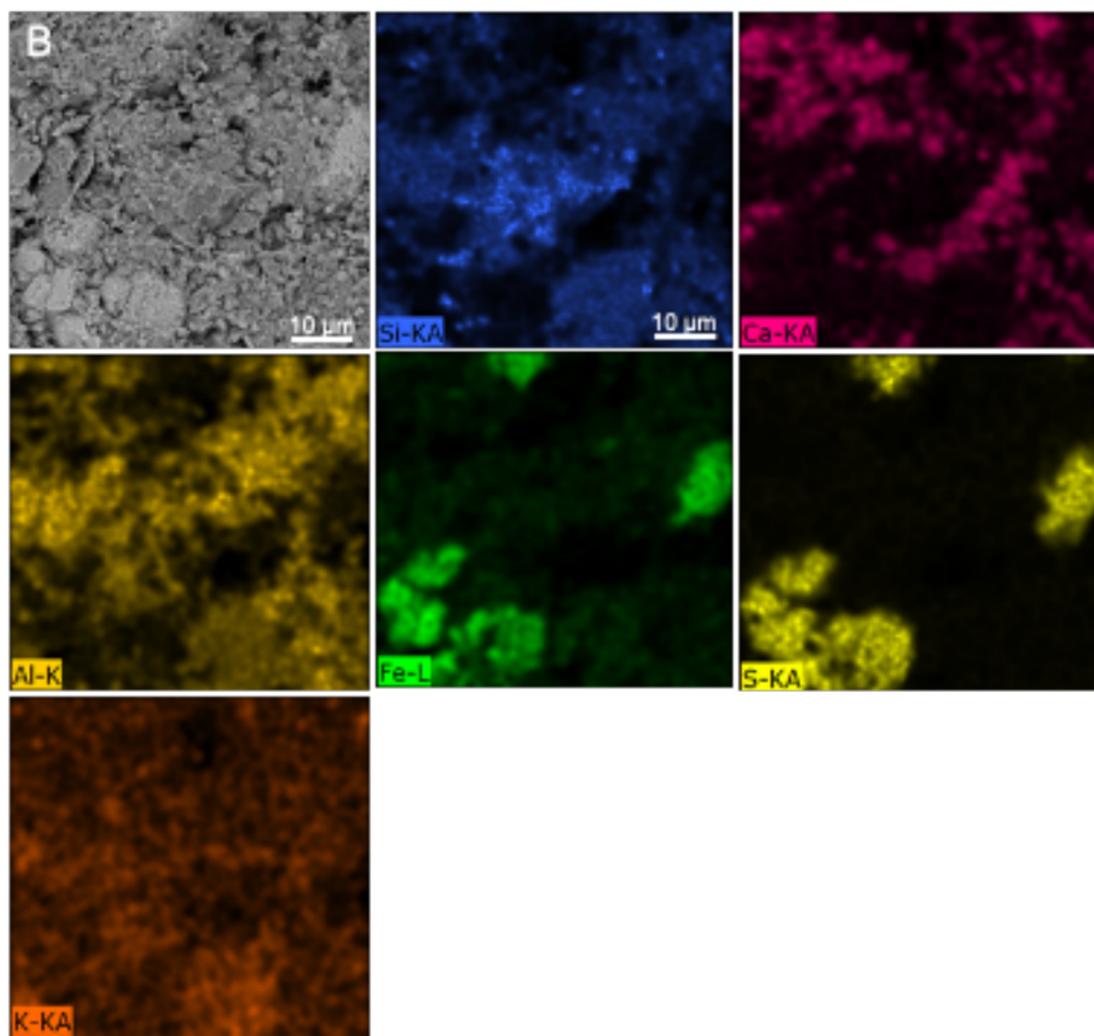
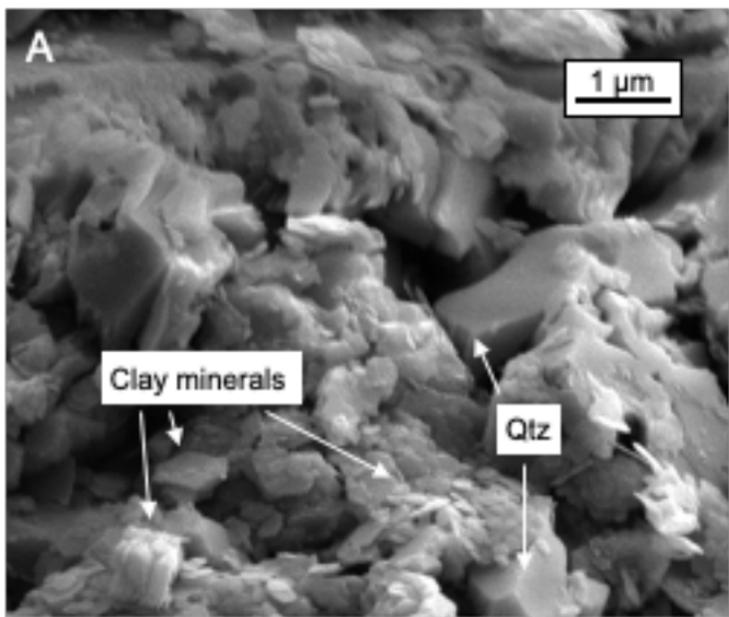
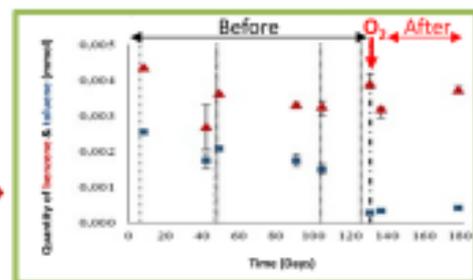
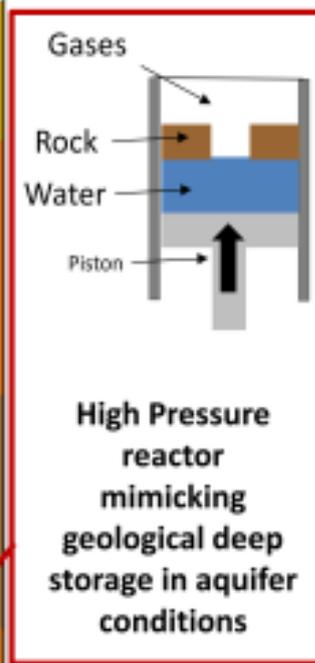
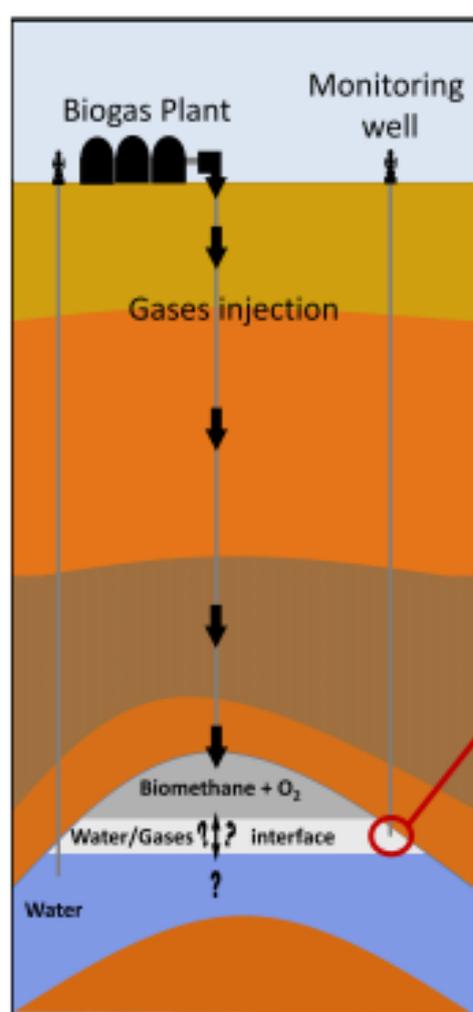


Table 1 – Physico-chemical parameters and compounds of the formation water sampled from the deep aquifer (Ab_L_1 site), analyzed at atmospheric pressure.

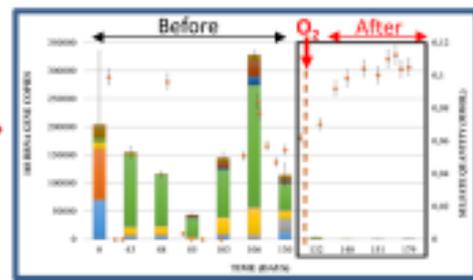
Physico-chemical Parameters	Value	Unit
Pressure	60	Bar
Temperature	36	°C
pH	7.6	
Redox potential	-259	mV
Conductivity at 25 °C	330	μS_cm
Organic carbon	0.38	mg.L ⁻¹
Compounds		
Chloride	0.2475	mM
Fluoride	0.0043	mM
Nitrate	0.0034	mM
Nitrite	0.0005	mM
Phosphate	0.0041	mM
Sulfate	0.0835	mM
Carbonate	0.0083	mM
Bicarbonate	3.0116	mM
Calcium	1.1884	mM
Iron	6.6122	μM
Dissolved iron	5.4486	μM
Magnesium	0.2465	mM
Potassium	0.1556	mM
Sodium	0.4930	mM

Table 2 – Standard deviation related to the 16S rRNA transcripts.

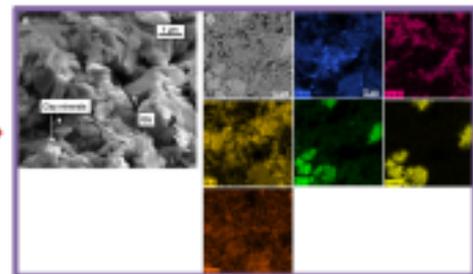
Time (Days)	Standard Deviation (16s rRNA transcripts)
0	1.14E+5
43	5.87E+5
48	5.32E+4
85	4.05E+3
103	2.01E+3
107	3.65E+3
130	6.19E+3
179	1.71E+3



Physico-chemistry



Microbiology



Imagery - Mineralogy