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# Microfluidics for studying the deep underground biosphere: From applications to fundamentals

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12 **Keywords: Deep underground ecosystems, microfluidics, micromodels, microorganisms, high**  
13 **pressure**

14 **Abstract**

15 In this review, selected examples are presented to demonstrate how microfluidic approaches can be  
16 utilized for investigating microbial life from deep geological environments, both from practical and  
17 fundamental perspectives. Beginning with the definition of the deep underground biosphere and the  
18 conventional experimental techniques employed for these studies, the use of microfluidic systems for  
19 accessing critical parameters of deep life in geological environments at the microscale is  
20 subsequently addressed (high pressure, high temperature, low volume). Microfluidics can simulate a  
21 range of environmental conditions on a chip, enabling rapid and comprehensive studies of microbial  
22 behavior and interactions in subsurface ecosystems, such as simulations of porous systems,  
23 interactions among microbes/microbes/minerals, and gradient cultivation. Transparent microreactors  
24 allow real-time, noninvasive analysis of microbial activities (microscopy, Raman spectroscopy, FTIR  
25 microspectroscopy, etc.), providing detailed insights into biogeochemical processes and facilitating  
26 pore-scale analysis. Finally, the current challenges and opportunities to expand the use of  
27 microfluidic methodologies for studying and monitoring the deep biosphere in real time under deep  
28 underground conditions are discussed.

29 **List of acronyms**

30 EOR: Enhanced Oil Recovery

31 FTIR: Fourier transform infrared

32 MEOR: Microbial Enhanced Oil Recovery

33 PMMA: Poly(methyl methacrylate)

34 PDMS: Polydimethylsiloxane

35 MICP: Microbiologically induced calcite precipitation

36 SLiMES: Subsurface Lithoautotrophic Microbial Ecosystems

37 X-ray CT: X-ray computed tomography

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## 38 1 Introduction

39 The depths of our planet, particularly in continental systems, have gained increasing attention, as  
40 they have been suggested to be the home of millions of undiscovered microbial organisms (Bar-On,  
41 2018; Magnabosco, 2018). This intraterrestrial world has rarely been investigated, especially for  
42 technical and economic issues, although several national and international multidisciplinary  
43 programs have emerged to facilitate exploration and determine the impact of autochthonous  
44 microorganisms on deep geochemical cycles, such as the International Ocean Discovery Program, the  
45 International Continental Scientific Drilling Program or the Deep Carbon Observatory's Census of  
46 Deep Life (Colwell, 2013; Pedersen, 2000; Fry, 2008; Edwards, 2012; McMahon, 2014; Escudero,  
47 2018; Mangelsdorf, 2010).

48 ***The deep underground biosphere and its investigation.*** In addition to the absence of sunlight, the  
49 key parameter driving the definition of deep environments—to distinguish them from classical  
50 surface environments—is pressure (typically  $p > 10$  MPa). The lithostatic pressure can exhibit strong  
51 lateral and depth heterogeneity, such as in three typical geological formations: (i) unmineable coal  
52 reservoirs, (ii) oil reservoirs, and (iii) deep saline aquifers (De Silva, 2015; Jayasekara, 2020; Liu,  
53 2020). Investigating the role of the deep biosphere in global cycles and its interaction with human  
54 activities is not straightforward. These environments are extremely difficult to access and require  
55 scientific drilling, the implementation of protocols to recover characteristic samples or sampling  
56 campaigns in mines such as those in South Africa or China (Takai, 2001; Zhang, 2005; Mangelsdorf,  
57 2010; Ranchou-Peyruse, 2023). The pioneers of high-pressure (HP) microbiology developed devices  
58 to sample and investigate deep-living organisms at the laboratory scale, including high-pressure flow  
59 environments (Jannasch, 1996; Parkes, 2009). Such experimental breakthroughs have demonstrated  
60 the extended pressure and temperature limits of life (Takai, 2008; Yayanos, 1981; Zeng, 2009) and  
61 offered a better understanding of microbes' adaptation to harsh environments, including  
62 biophysiological mechanisms (Cario, 2015; Cario, 2016; Amrani, 2016). However, the current  
63 equipment mostly uses large volumes and cannot easily accommodate *in situ* characterization  
64 techniques. Thus, Garel and colleagues (2019) developed an equipressure transfer procedure *via*  
65 high-pressure bottles (stainless steel coated with PEEK), allowing subsampling to carry out a series  
66 of experiments. This means that conventional HP microbiology methods (phenotyping,  
67 characterization, etc.) are time-consuming and often favor well-known fast-growing organisms that  
68 outcompete others in culture. In addition, sample decompression, which is required for microbial

69 characterization, is a common process that can skew community diversity and structure, and isolate  
70 physiology (Jayasekara, 2020). The main challenge in reproducing acceptable deep environmental  
71 conditions in the laboratory is therefore the need for suitable experimental tools in terms of the  
72 observation scale and pressure/temperature range.

73 ***The rise of microfluidics.*** Microfluidics is a field of research that addresses the precise manipulation  
74 of fluids at small scales, typically from microliter to picoliter volumes. This interdisciplinary area  
75 combines principles from physics, engineering, chemistry, and biology to create devices known as  
76 "lab-on-a-chip" systems. Microfluidic devices utilize micron-sized channels, chambers, and valves to  
77 integrate various functionalities, such as mixing, separation, and analysis, into compact microscale  
78 platforms, typically of the size of a credit card (see Figure 1). By enabling precise control over fluids  
79 at the microscale, microfluidics has revolutionized experimentation and analysis, opening new  
80 avenues for scientific discovery and technological innovation. Microfluidic tools have been  
81 extensively used over the past 20 years for microbiology (Duncombe, 2015; El-Ali, 2006),  
82 thermodynamics (Pinho, 2014), hydrodynamics (Zhang, 2018), materials synthesis (Marre, 2010) and  
83 chemistry (Hartman, 2009; Jensen, 2014). Microfluidic tools have inherent advantages, such as low  
84 sample consumption, faster equilibrium times, better control of the experimental conditions,  
85 flexibility in terms of designs for recreating complex geometries (Morais, 2020) and high-throughput  
86 capabilities. For example, we recently demonstrated the use of confined microfluidic chambers for  
87 HP microbial phenotyping (Cario, 2022) under anoxic conditions, whereas others used similar  
88 approaches to simulate and observe fluid mixtures in real time to understand precipitation  
89 mechanisms during pH variations in the case of hydrothermal vents on the ocean floor (Weingart,  
90 2023). On the basis of these characteristics, microfluidics has largely been involved in accelerating  
91 discoveries in microbiology, although far focusing almost exclusively on biomedical and health  
92 applications along with investigations of human cells. These studies considered only pressure and  
93 temperature conditions close to ambient conditions with "conventional" laboratory model  
94 microorganism strains and were mostly interested in investigating fundamental microbial  
95 mechanisms and their responses to stimuli. Only a few reports exist on applications of microfluidics  
96 for environmental microbiology, such as planktonic and microalgal research (Wang, 2020; Girault,  
97 2019), but again, these reports are close to ambient conditions. Nevertheless, given the flexibility  
98 provided by photolithography, it is rather straightforward to reproduce "micromodels" mimicking the  
99 porous geometry of deep underground environments, which has driven the use of microfluidics to  
100 investigate—at the laboratory scale—subsurface processes, particularly those linked with human

101 activities (geological CO<sub>2</sub> storage, EOR, etc.) (Lifton, 2016; Morais, 2016; Morais, 2020). The  
102 resulting microfluidic devices generally consist of 2D patterns extruded in the third shallower  
103 dimension on a substrate (glass, silicon, PMMA (poly(methyl methacrylate)) or cast on a mold  
104 (polydimethylsiloxane (PDMS)) and chemically or physically sealed with a transparent material  
105 (Morais, 2020). However, it is important to note that access to 3D micromodels is also possible  
106 owing to multiple etching steps, leading to multidepth geometrical profiles (Park, 2015).  
107 Additionally, minerals can also be inserted inside microchannel geometries to reproduce full 3D  
108 porous media (Bowden, 2016). These micromodels have demonstrated that they can be powerful  
109 tools to complement more conventional experimental methods, such as core flood experiments,  
110 particularly when they are compatible with experimental work performed under harsh conditions. As  
111 an illustration, Table 1 summarizes some advantages and limitations of conventional and  
112 microfluidic approaches for investigating the deep underground biosphere.

113 Hence, investigating the biological processes in micromodels enables real-time observations and  
114 monitoring of microorganism metabolism in realistic microporous media (Almeida, 2018; Lee, 2017;  
115 Sun, 2011; Tang, 2014; Xu, 2013).

116 On the basis of these advantages, through selected examples, current studies concerning the use of  
117 micromodels for studying the interactions between the deep biosphere and a selection of human  
118 activities in deep geological porous reservoirs are presented in this review. Then, several challenges  
119 and opportunities related to the utilization of microreactors for such studies are discussed. High-  
120 pressure and high-temperature transparent microfluidic systems are powerful tools whose use  
121 deserves to be developed to simulate exploited scenarios and generate missing experimental data  
122 for large-scale modeling of the evolution of exploited reservoirs and address certain fundamental  
123 questions related to deep underground life (Figure 1).

## 124 **2- Microfluidics approaches for evaluating the impact of microbiology on deep underground** 125 **anthropogenic activities.**

126 Micromodels have been extensively used to investigate several bioprocesses occurring in geological  
127 environments at the pore scale. These micromodels provide critical physicochemical information,  
128 which are some keys to driving the efficient injection and recovery of fluids, both in deep geological  
129 reservoirs and in geochemical reactions, reactive flows or biofilm formation and monitoring.

130 We discuss hereafter some selected examples of the use of microfluidics and micromodels for  
131 accessing a deeper understanding of how the deep biosphere interacts with human exploitation of  
132 geology underground.

## 133 **2.1 Biosurfactant generation influence on interfacial phenomena**

134 Microbial enhanced oil recovery (MEOR) is a technology developed to accelerate and increase  
135 underground oil recovery via the use of microorganisms. A combination of various mechanisms  
136 improves the sweep and displacement efficiency. In the literature, the following mechanisms are  
137 commonly quoted and investigated:

- 138 - An improvement in mobility due to changes in oil and water/brine viscosities is mainly  
139 related to the dissolution of gases (CO<sub>2</sub>, H<sub>2</sub>, N<sub>2</sub> and CH<sub>4</sub>) produced by microbial metabolism  
140 (Alkan, 2014; Sugai, 2014).
- 141 - An increase in microscopic sweep efficiency due to changes in interfacial tension (Armstrong,  
142 2012; Paulsen, 1999; Nourani, 2007) and wettability (Polson, 2010; Armstrong, 2012; Al-  
143 Raoush, 2009) decreases capillary forces.
- 144 - Biofilm formation (bio-plugging) reduces the permeability in high-permeability zones or  
145 fractures (Kaster, 2012; Jenneman, 1984; Gray, 2008; Karambeigi, 2013) and causes the  
146 corrosion of pipelines (production of H<sub>2</sub>S) in some cases (Zhou, 2022).
- 147 - The production of acids can lead to rock dissolution.

148 The change in wettability and the reduction in interfacial tension (IFT) caused by biosurfactants are  
149 key parameters for MEOR processes. They strongly affect the fluid displacement mechanisms at the  
150 microscale, and an increase of three orders of magnitude is necessary to displace oil from capillaries  
151 of 10–100 μm (Karimi, 2012; Reed, 1977; Crescente, 2008; Kowalewski, 2006). Hence,  
152 microfluidics may help overcome the lack of microscale information. Indeed, over the past 30 years,  
153 microfluidics approaches have proven that they are indubitably suitable for studying wettability  
154 effects on drainage or imbibition for problematic EOR or geological CO<sub>2</sub> storage and sequestration  
155 investigations (Bora, 2000; Cao, 2019; Gerami, 2018; Lifton, 2016; Nguyen, 2002; Sohrabi, 2001;  
156 Jamaloei, 2009).

157 Bryant *et al.* studied the feasibility of enhancing oil recovery via the injection of microorganisms into  
158 a reservoir (Bryant, 1990; Bryant, 1988; Buciak, 1996). These microorganisms provide amphiphilic  
159 molecules that act as biosurfactants, leading to the detachment of the oil from the reservoir rocks.

160 Studies have shown that *Bacillus licheniformis*, which was isolated from a pristine oilfield, was the  
161 most effective (da Cunha, 2006). Three decades later, Afrapoli *et al.* explored the performance and  
162 mechanisms of model bacterial strain flooding at the pore scale (Afrapoli, 2012; Afrapoli, 2011).  
163 They investigated the improvement in oil (dodecane) recovery essentially due to an alkane-oxidizing  
164 *Rhodococcus* sp. strain 094 in glass micromodels with three different configurations (the ratio of the  
165 pore diameter to the pore throat dimension) and different wettabilities (water wet or oil wet). The  
166 emulsification process occurs while the cells are growing exponentially and involves IFT reduction,  
167 wettability changes and pore blocking mechanisms (Bredholt, 1998; Crescente, 2008).  
168 Microorganisms produce biosurfactants to access hydrophobic compounds, effectively reducing  
169 interfacial tension (IFT), altering wettability, modifying flow patterns, and decreasing oil viscosity  
170 (Soudmand-asli, 2007). Indeed, a study with a glass micromodel showed that the use of a surfactant  
171 produced by *B. subtilis* led to additional oil recovery (Hadia, 2019). The presence of bacterial cells  
172 appears to amplify the oil recovery effect of biosurfactants, underscoring the increasing importance  
173 of interactions between microorganisms and their environment. White and colleagues published work  
174 on the interaction of a single oil droplet and bacterial strains *via* a microfluidic platform, *ecology-on-*  
175 *a-chip* (White, 2019). Owing to the layer-by-layer hydrophilic polyelectrolyte coating of the surface  
176 of the microchannel, an oil drop was immobilized for over a month. The authors observed the  
177 aggregate morphology as well as the interfacial response between the oil and the microbial  
178 environment. This approach represents a tremendous step forward in the matter of long-term  
179 observation of the MEOR process in microfluidics. While most MEOR studies in the literature are  
180 conducted under atmospheric pressure and oxic conditions, some strive to replicate actual  
181 environmental conditions more accurately. For example, Gaol and colleagues (Gaol, 2019) succeeded  
182 in investigating the pore-scale mechanisms of MEOR under relatively harsh processing conditions  
183 (37 °C, 0.6 MPa). Nonetheless, these conditions are far from realistic reservoir conditions, typically  
184 between 6 MPa and 15 MPa and between 30 °C and 80 °C (Morais, 2020; Morais, 2016). The  
185 development of HP microfluidics could help address this gap (Marre, 2010). Moreover, microfluidic  
186 approaches combined with image reprocessing now make it possible to monitor the growth of  
187 microorganisms in real time while tracking their behavior in relation to the studied activity in general  
188 (Strobel, 2023) and their interaction with oil droplets in the particular case of MEOR (Gaol, 2021).

189 In addition to planktonic microorganisms, biofilm formation and monitoring are critical parameters  
190 to investigate when considering deep underground utilization since they can severely change some  
191 operating parameters, such as injectivity and reservoir rock stability. Thus it is important to



192 investigate the interactions between the considered substrates and the microorganisms. Microfluidics  
193 has been used to recreate several geometrical parameters from porous media while allowing *in situ*  
194 observations. These observations can provide deeper insight into the behavior of biofilms in confined  
195 geometries, as discussed in the next section.

## 196 **2.2 Biofilm growth, metabolism, survival and degradation in confined environments**

197 In deep porous environments, microorganisms are known to grow mainly by attaching themselves to  
198 rock surfaces (Whitman, 1998; Bar-On, 2018; McMahon, 2014; Griebl, 2002). MEOR applications  
199 can create barriers through pore clogging, leading to a reduction in the permeability of the reservoir  
200 (Kim, 2000; Lappan, 1996). Conversely, investigations revealed that similar bioclogging mechanisms  
201 can contribute to decreases in porosity and permeability in geological CO<sub>2</sub> storage reservoirs  
202 (Glatstein, 2014; Mitchell, 2009; Thullner, 2002).

203 One of the pioneering works concerning biofilm processes at the microscale in porous media  
204 (Dunsmore, 2004) showed that, for the formation of a biofilm developed by cells of sulfate reducers  
205 (*Desulfovibrio* sp. EX265), a decrease in permeability was associated with biofilm growth. From that  
206 observation, correlations were established with the bioclogging of the pore space, which was  
207 previously detected at the core scale and field scale (Cunningham, 1991). Later, the same team  
208 coupled image analyses and chemical analyses to investigate the effects of nitrate treatment on  
209 oilfield microbial biofilms (Dunsmore, 2006). This time, they used glass capillary flow cells. They  
210 demonstrated the influence of nitrate treatment on sulfate reducer biofilms as well as changes in  
211 biofilm cell morphology. Additionally, chemical analyses revealed that the injection of nitrate ions  
212 into the flow cell changed the dominant metabolic process from sulfate to nitrate reduction. Another  
213 study including the use of a PDMS-based microfluidic channel revealed that the resistance of a  
214 biofilm developed by *Pseudomonas aeruginosa* was due mainly to the formation of stable  
215 extracellular polymeric substances, which provide a mechanical shield (Park, 2011).

216 Investigations at the microscale have been increasingly conducted to better understand how biofilms  
217 initiate their formation in different environments (Zhang, 2019) and how biofilms impact mineral  
218 surface properties, geochemistry and hydrodynamics in porous media (Liu, 2019). A recent study  
219 focused on sulfate reducer colonization in shale fractures, which promotes biosouring in oil and gas  
220 reservoirs (Zhou, 2022). In this context, a shale-based microfluidic flow cell reactor allows the  
221 quantification of both sulfate reduction rates and biomass growth while looking for biocide

222 inhibition. This experiment revealed that biocides had little effect on biomass removal and opened  
223 shale fractures, which could contribute to the development of mitigation strategies for preventing  
224 environmental impacts on underground processes. As micromodels provide access to direct  
225 observations of biofilm evolution at the microscale (Yawata, 2016; Lam, 2016), they allow full  
226 visualization of the environmental effects of nutrient concentration, cells, surface properties  
227 (wettability, IFT) or shear stress on these biological formations (Skolimowski, 2010; Wang, 2018;  
228 Wilkins, 2014; Zhang, 2019). These microscale approaches are already used to study the properties  
229 of biofilms, such as stress/growth curves or deformation and cell detachment, through  
230 microrheological studies (Klapper, 2002; Dunsmore, 2002; Stoodley, 2002; Stoodley, 1999; Billings,  
231 2015; Liu, 2023). Undeniably, these tools provide the ability to observe different phenomena in detail  
232 and collect quantitative data from the growth of the biofilm through the effects of starvation  
233 conditions (Kim, 2000), the estimation of the intrinsic permeability of the biofilm itself  
234 (Hassannayebi, 2021), or biodegradation in porous media at the pore scale (Vayenas, 2002).

235 Another interesting aspect of microfluidic approaches is the ability to decouple complex  
236 simultaneous effects such as the effect of nutrient flow velocity and the impact of shear stress on  
237 biofilm formation (Liu, 2019). Authors performed a hydrodynamic study on biofilm growth (from  
238 *Thalassospira* sp.) and experimented with biofilm detachment with a T-shaped microchannel. Direct  
239 observation through a glass microsystem allowed spatial localization of biofilm accumulation and  
240 evolution of the adhesive strength with various flow velocities and nutrient concentrations. This  
241 permitted (i) the determination of the conditions that lead to a plugging effect and (ii) the estimation  
242 of the capacity of biofilm formation to reduce the permeability of model deep underground porous  
243 media.

244 Sygouni and coauthors (Sygouni, 2016) studied the impact of CO<sub>2</sub> injections on a *Pseudomonas*  
245 *putida* biofilm in a micromodel. After inoculation in a glass/glass pore network and over the course  
246 of 18 days, nutrient broth was injected into the micromodel to induce the growth of the biofilm. A  
247 series of CO<sub>2</sub> injections were subsequently performed at different times. They observed a pH  
248 reduction immediately after each CO<sub>2</sub> injection, leading to cell stress and inducing partial detachment  
249 of the *P. putida* biofilm. However, this gas injection had only a temporary effect on the biofilm, as  
250 evidenced by the regrowth after the completion of each injection. As the nutrient flow compensates  
251 for the porous media and CO<sub>2</sub> is consumed by various buffering reactions, its concentration then  
252 decreases, and biofilm saturation progressively reaches its initial value. The same observation was

253 made for the permeability, which recovered quickly from its initial low value (due to the presence of  
254 the biofilm at the beginning of the experiment). The study highlighted that biofilm age is crucial,  
255 with older biofilms being denser and more resistant to degradation.

256 In a study on MEOR efficiency with microorganisms and their associated bioproduced surfactants,  
257 biofilm formation was also observed (Armstrong, 2012). In a 2D transparent porous medium,  
258 changes in the pore morphology through the propagation of the biofilm and the redirection of the  
259 flow were observed, creating a new preferential path pattern. This investigation confirmed the  
260 suitability of 2D porous media for studying the hydrodynamic impacts of biofilms (Aufrecht, 2019).

261 In addition to microbial production, the hydrodynamics of a whole geological reservoir can also be  
262 impacted by microbial activity. Indeed, chemical gradients and chemotaxis have been shown to  
263 influence the dispersion of degrading microorganisms within a porous matrix (de Anna, 2021; Wang,  
264 2012; Wang, 2015; Wang, 2016).

### 265 **2.3. Mineralization induced by microorganisms at the microscale.**

266 Biomineralization is yet another effect of deep underground microorganisms, especially in storage  
267 applications, that can affect the evolution of geological reservoirs, and it has been observed at the  
268 core and field scales that the precipitation of carbonates through biomineralization metabolism leads  
269 to clogging in porous reservoirs (Dupraz, 2009; Mitchell, 2010; Fujita, 2008). Microfluidic  
270 technologies have the ability to perform biomineralization experiments in model porous media and  
271 allow direct observation of crystal growth during microbiologically induced calcite precipitation  
272 (MICP) processes (Singh, 2015; Wang, 2019; Wang, 2019; Wang, 2020). Several studies have shown  
273 that quantifying biomineral precipitation at the pore scale *via* image processing is possible (Kim,  
274 2018; Kim, 2020; Xiao, 2021; Xiao, 2024). These findings showed that characterizing biomineral  
275 precipitation in porous media is complex because of the involvement of multiphase reactive transport  
276 and interactive processes (Dejong, 2013; Hommel, 2016). For example, the nucleation and growth of  
277 crystals as well as the quantification of the crystal kinetics were performed in a Y-shaped sand-  
278 containing microchip. Such biomineralization process monitoring at the pore scale provides new  
279 insights for optimizing underground processes in geotechnical engineering, such as biocementation  
280 (Zheng, 2023; Elmaloglou, 2020). MICP experiments carried out under high-pressure conditions  
281 (100 bar) with *Sporosarcina pasteurii* resulted in lower CaCO<sub>3</sub> crystallization yields than those at  
282 atmospheric pressure (Liu, 2023), possibly because of the lower activity of the considered model

283 bacterium induced by HP conditions. For those interested in more information on MICP-based  
284 microfluidic techniques and modeling results, we recommend consulting the review by Xiao *et al.*  
285 (Xiao, 2023).

286 HP studies are still scarce, but integrating them with conventional HP microbiology tools could offer  
287 new insights and accelerate discoveries in this field, as discussed in the next section.

### 288 **3- Challenges, opportunities and limitations**

289 Many microbiology laboratories have equipment for handling strictly anaerobic microorganisms  
290 (anaerobic chambers) and culturing them under controlled atmospheres at near-atmospheric pressure.  
291 However, one of the main difficulties faced by microbiologists specializing in deep environments is  
292 working safely under HP conditions, especially when working with flammable (CH<sub>4</sub>, H<sub>2</sub>), toxic and  
293 corrosive gases (CO<sub>2</sub>, H<sub>2</sub>S). Many microbiology laboratories cannot accommodate classical HP  
294 reactors (hundreds of milliliters to a few liters), mainly because of space, safety (explosion, gas  
295 leaks) and cost. Microfluidic tools can help overcome these constraints along with the possibility of  
296 performing experiments with high reproducibility given the improved control of the operating  
297 parameters compared with large-scale experiments and the ability to work at the scale of the  
298 microenvironments in which the microorganisms evolve. Microfluidic cells can be manipulated  
299 inside an anaerobic chamber to introduce anaerobic microorganisms. The cells are then taken out to  
300 be connected to the HP pump containing the strict volume of fluids required for the experiment. In  
301 addition, it is possible to screen various conditions simultaneously in a single experiment, generating  
302 high throughput with a low sample volume (Xu, 2017; Tang, 2014; Gantz, 2022). For example, the  
303 possibility of observing the effects of many chemical and physical variables simultaneously has not  
304 yet been fully exploited and could extend the scope of possibilities. Moreover, the ability to control  
305 gradients and dissociate the effect control separately for each experimental parameter at very small  
306 scales will allow strong multiplication of intercomparable results in a single experiment, as recently  
307 demonstrated for microbial phenotyping under pressure (Cario, 2022). Finally, the use of  
308 microfluidics lends itself naturally and easily to the study of organisms evolving in strictly anoxic  
309 conditions since such experiments can be perfectly well controlled (Mohr, 2010; Dickson, 2019;  
310 Cario, 2022).

311

### 313 **3.1. Fast screening challenge under harsh conditions at the microscale**

314 **On-chip multiple experimental conditions.** It is possible to generate temperature gradients in  
315 microreactors to study, for example, the optimal culture conditions of a microbial strain, understand  
316 the behavior of a microbial community, or even perform the isolation of microorganisms from  
317 complex communities originating from the deep subsurface. Concentration gradients can also be  
318 easily generated on a chip to evaluate, for example, their influence on biofilm growth, attachment and  
319 interaction with mineral surfaces (Jeong, 2014; Yawata, 2014; Zhang, 2019). These gradient  
320 approaches can also be applied to other physicochemical parameters, such as salinity, pH, and  
321 molecules of interest (chemotaxis, toxicity), hence providing ways to perform multiple experimental  
322 conditions in a single experiment. Such methodologies can therefore speed up the rate at which deep  
323 underground related experiments can be performed.

324 **High-pressure/high-temperature microfluidics.** Conventional transparent micromodels cannot  
325 withstand high-pressure conditions. However, it is possible to work at high pressures and  
326 temperatures with micromodels whose channels are etched in mechanically resistant materials such  
327 as silicon, onto which borosilicate glass slides are bonded (Marre, 2010; Trachsel, 2008). The use of  
328 such microfluidic reactors for microbiology studies under harsh conditions has already been  
329 demonstrated (Cario, 2022), but these require reflected light microscopy, which is not ideal for  
330 microbiology. However, such a setup precludes that the use of transmission optical microscopy  
331 would be efficient when coupled with transmitted light microscopy for optimum observation of  
332 microorganisms. While glass–glass microreactors (Dietrich, 2005) have demonstrated HP capabilities  
333 up to 20 MPa or more (Tiggelaar, 2007), they are not able to cover a large range of conditions  
334 representative of the deep subsurface and are only transparent in the visible range. However, it is now  
335 possible to produce full sapphire microreactors capable of working at pressures of up to 80 MPa  
336 (Marre, 2021) while exhibiting transparency from UV to mid-IR, opening opportunities for further  
337 investigations under larger parameter window conditions. Hence, HP-compatible microfluidics tools  
338 exist that can be used in combination with more conventional HP reactors for studying the deep  
339 underground biosphere. Both experimental scales are equally important for investigating  
340 multiparameter biogeochemical processes from the pore scale to the core scale and further at the  
341 reservoir scale. Similarly, while microfluidics allows fast parameter screening, conventional larger

342 scale reactors can generally achieve better quantitative fluid analysis because of the large processed  
343 volumes.

344 **In situ live characterization of microbial activities in microfluidics.** Working with transparent  
345 micromodels at a wide range of wavelengths opens up a wide range of possibilities for real-time  
346 analyses, starting with all types of microscopy: confocal, fluorescence, Raman spectroscopy (Liu,  
347 2012; Ochoa-Vazquez, 2019), FTIR microspectroscopy (Perro, 2016) and X-rays for analysis  
348 (Beuvier, 2015) and imaging (Morais, 2023). The combined use of such characterization techniques  
349 allows access to critical information on local biogeochemical processes and microbial metabolism  
350 without any external perturbation of the studied reactive medium. For example, Raman spectroscopy  
351 can be used to selectively identify different strains without any fluorescent labeling (Dhankhar, 2021;  
352 Rebrosova, 2022; Shakeel, 2022; Lister, 2022). Similarly, live imaging of living cells can be obtained  
353 by coupling FTIR microspectroscopy with microfluidics (Loutherback, 2016; Vaccari, 2012). The  
354 advantage of FTIR over Raman is that imaging can be performed (in contrast to dot-by-dot mapping),  
355 thus providing instant localized characterization. For example, Birarda and colleagues (Birarda,  
356 2016) developed a low-cost microfluidic platform (IR-Live) compatible with synchrotron FTIR  
357 imaging to study living eukaryotic cells. On the basis of the specific vibrational footprint of  
358 molecules, they were able to identify and localize proteins and lipids without any preliminary  
359 labeling. This technique is perfectly well suited for monitoring microbial activities in biofilms  
360 (Holman, 2009; Pousti, 2018) and could also be coupled with Raman spectroscopy, as proven  
361 concepts have already been developed with other methodologies (Gieroba, 2020; Muhamadali, 2015;  
362 Lima, 2022; Rohman, 2019) and could easily be adapted to microfluidic experimentation.

### 363 **3.2. Accessing realistic geometries and mineralogy on a chip**

364 Microfluidic experiments performed to study applications related to the utilization of deep  
365 underground provide excellent control and monitoring of operating parameters such as fluid velocity  
366 and composition, temperature and pressure (Morais, 2016). Additionally, micromodels enable  
367 laboratory simulations of the microporosity conditions characteristic of porous rocks encountered in  
368 deep environments, such as aquifers or hydrocarbon reservoirs. Owing to the development of  
369 microfabrication techniques, all types of designs can be created, providing a flexible way to meet the  
370 needs of mimicking realistic environments that are site-specific (see Section 2 from (Morais, 2020)).  
371 Most of the microreactors developed thus far for deep underground studies can be seen as 2D porous  
372 structures, which are perfectly suitable as “micromodels” to describe and study the biophysico-

373 chemical mechanisms occurring inside; however, the third dimension is very small, and some  
374 researchers often raise concerns about the applicability of the results obtained in 2D compared with  
375 the real 3D world, which includes reactive fluid flow, dissolution/precipitation mechanisms, biofilm  
376 development, etc. Recent developments have allowed the access of 2.5- or 3D structures on a chip,  
377 which help account for vertical flows (similar to core flood experiments), but in a controlled manner  
378 owing to microfabrication protocols (Park, 2015). Of course, not all microbiologists have the know-  
379 how and equipment to produce such micromodels. However, an increasing number of companies are  
380 offering products that can be adapted for sale to microbiologists and do not necessarily require  
381 advanced competencies. When studying microbial processes in porous media, surface chemistry is  
382 equally critical to account for since those interfaces are known to regulate mainly the exchanges and  
383 compositions of the fluids that surround the microbial cells. Hence, the use or inclusion of actual  
384 rocks and mineral surfaces that perfectly mimic the underground pore system is essential to obtain  
385 materials as close as possible to the fluid–mineral surfaces encountered in the target environments.  
386 Recent developments in microfabrication and surface chemistry in microreactors promise to reach  
387 this goal. The microporous network can now be reproduced in actual rocks (Hauge, 2016; Singh,  
388 2017). The surface properties of micromodels can thus be modified and tuned with great flexibility,  
389 for example, by functionalization with molecules (Song, 2015) or minerals such as crystallized  
390 calcite and others (Yoon, 2012), or by directly fabricating micromodels “out-of-the-rock” (Song,  
391 2014), which can exhibit pressure resistance up to 100 bar (Porter, 2015).

### 392 **3.3. Some examples of current scientific challenges**

393 Microfluidic tools have the potential to revolutionize a wide range of research into microporous  
394 ecosystems, whether terrestrial or intraterrestrial. High pressure plays an obvious role in the  
395 physiology, morphology and behavior of prokaryotes (Abe, 2007). Thus, an increase in pressure has  
396 already been shown to be responsible for the decrease in acetate production to the benefit of formate  
397 by *Clostridium ljungdahlii* (Oswald, 2018). High-pressure conditions have repeatedly been shown to  
398 produce deformations associated with cell division problems (Welch, 1993; Ishii, 2004). The use of  
399 transparent HP micromodels would enable these adaptations to be observed in real time. The  
400 following questions and current challenges are just a few examples of the approaches that could  
401 benefit from microfluidic technologies to study life from the molecules to microbial communities and  
402 societal applications, although this list is by no means exhaustive.

403 **Pore-scale view of geological CO<sub>2</sub> and H<sub>2</sub> storage.** CO<sub>2</sub>, as a waste, and H<sub>2</sub>, as a strategic energy  
404 source, are key molecules to be stored deep underground, benefitting from the large volume offered  
405 by such an environment (Haddad et al., 2022). However, both molecules can directly interact with  
406 deep environments, either through geochemical or biogeochemical reactions. This means that for  
407 such applications, it is important to learn from the benefit of scientific feedback concerning what  
408 could be stored at each specific selected geological site and what could be the result of such storage  
409 (Ranchou-Peyruse *et al.*, 2019). While local measurements can be made concerning site-specific  
410 properties in terms of geophysical and geochemical properties, performing realistic storage tests at  
411 the field scale is quite difficult, both for technical and economic reasons. Hence, laboratory-scale  
412 approaches could help obtain insights into further industrial strategies. In that context, microfluidic  
413 experiments exhibit several interesting specificities, which could be complementary to conventional  
414 core flood experiments, such as direct in situ visualization and biogeochemical characterization of the  
415 ongoing phenomena with relatively simple laboratory equipment. Some papers concerning geological  
416 CO<sub>2</sub> storage (Morais, 2016; Morais, 2020) and, more recently, H<sub>2</sub> storage (Lysyy, 2023; Liu, 2023;  
417 Lysyy, 2024) have been published, resulting in a pore-scale view and investigations of the  
418 geochemical and biochemical effects, particularly the effects of deep underground microorganisms.  
419 Similarly, another study reported the use of microfluidic approaches for the simulation of alkaline  
420 vents (Weingart et al., 2023). In all these studies, the interest of coupling conventional and  
421 microfluidic approaches to investigate energy or waste underground storage is evident since the  
422 obtained data can be combined for a multiscale view of the mechanisms. From that point, typical  
423 geochemical reactions such as carbonation or serpentinization could be studied on a chip. Indeed, in  
424 the presence of water and at specific temperatures, iron-bearing minerals can lead to geochemical  
425 reactions along with the production of H<sub>2</sub>. Olivine, greigite, magnetite and awaruite can act as  
426 catalysts for the subsequent formation of simple carbon-containing molecules (acetate, formate,  
427 pyruvate, and methanol) (Preiner, 2018; Preiner, 2020). These simple molecules can in turn feed  
428 entire microbial ecosystems and are at the origin of the SLiME concept for subsurface  
429 lithoautotrophic microbial ecosystems on the basis of H<sub>2</sub> and CO<sub>2</sub> (Stevens, 1995; Fry, 1997;  
430 Kotelnikova, 1997; Chapelle, 2002; Haveman, 2002; Takai, 2003; Lin, 2005; Basso, 2009; Crespo-  
431 Medina, 2014). Moreover, such ecosystems can interact strongly with the injection of gas feedstocks,  
432 which are either anthropogenic waste (CO<sub>2</sub>) or energetic resources (natural gas, H<sub>2</sub>, etc.). Hence,  
433 microfluidics approaches can help mimic the geological environment at the laboratory scale to  
434 evaluate the impact of such injection on the local biosphere at the pore scale (consumption, reservoir  
435 stability, biofilm formation, variation in injectivity, process integrity, etc.).



436 **Microfluidics fast screening approaches for database generation for numerical modeling and**  
437 **scale-up.** Another gap is the need to experimentally assess the scaling-up effect between  
438 micromodels and larger and more traditional set-ups. In fact, the numerous studies presented here  
439 aimed to connect the conclusions from microfluidic studies with other observations in the laboratory  
440 or even at the reservoir scale. Modeling can partially address that need but does not satisfy the  
441 necessary experimental data and demonstrations. This is where microfluidics approaches can play a  
442 critical role. Indeed, one of the major interests with microfluidic experimentation concerns the ability  
443 to perform several experiments in a single run. This method has already been applied to  
444 crystallization (Bhattacharya, 2020; Chauhan, 2023), drug screening (Sun, 2019; Cui, 2019), organic  
445 chemistry, synthetic and structural biology (Kwon, 2023; Hansen, 2003) and microorganism  
446 cultivation (Cario, 2022; Watterson, 2020).

447 In addition, numerous replicates can be realized on the same system, making it possible to envision  
448 more robust approaches supported by statistical processing while working under exemplary safety  
449 conditions. Hence, microfluidics has the ability to perform fast screening for generating multiple  
450 data, which could be implemented in machine-learning approaches to predict biogeochemical  
451 reactivity, permeability variation or biofilm development. All these aspects can further be used in  
452 numerical modeling from the pore scale to the reservoir scale, although the scale-up process is still  
453 not straightforward. To evaluate separate parameters to assess thermodynamics and kinetics,  
454 micromodels (on-chip porous media) can also be used to evaluate the impact of the deep biosphere  
455 on geological processes. This can be achieved by designing and microfabricating microreactors that  
456 mimic specific storage locations and/or different scenarios. For example, transparent high-pressure  
457 micromodel approaches coupled with X-ray imaging characterization, such as X-ray laminography  
458 (Morais, 2023), would enable a more detailed study of geochemical reactions under conditions  
459 simulating deep environments (pressure and temperature), providing additional information to more  
460 conventional methodologies based on core flooding coupled with X-ray CT (Minto, 2017; Davit,  
461 2011).

462 **Microbial interactions (microbe–mineral surface and microbe–microbial interactions).** Within  
463 the deep subsurface, microorganisms interact with their environment from both biological (cell-to-  
464 cell) and mineral (attachment and colonization) points of view. Getting access to such interactions at  
465 small scales can be performed with microfluidics. A recent study investigated the effects of exposure  
466 to short, low-carbon-weight compounds on biofilm formation on mica surfaces (Nuppenen-Puputti,

2023). The effects of the conditions tested (addition of methane, methanol and acetate) on the taxonomic diversity of the communities were then analyzed via high-throughput sequencing approaches performed on both water and rock samples. A complementary culturing approach using a mica-filled micromodel (Bhattacharjee, 2022) enabled real-time observation of the formation and/or disintegration of the biofilm formed as a function of the molecules injected to quantify microorganisms or to monitor the behavior of sessile and planktonic microorganisms. Similarly, competition or cooperation phenomena can be studied throughout incubation, depending on the parameters applied (substrate concentrations, flow rates, etc.). For example, Kubik and Holden (Kubik, 2023) studied the competition between different hydrogenotrophs belonging to three different species. The aim of this study was to understand the effects of H<sub>2</sub> concentrations on the metabolism of these microorganisms, particularly to demonstrate that these microorganisms redirected their redox reactions from CH<sub>4</sub> and H<sub>2</sub>S production to biomass production at lower H<sub>2</sub> concentrations. Axenic cultures and batch cocultures were generated at various incubation temperatures. Using existing microfluidic tools, it would be possible to create a temperature gradient (Mao, 2002) along with cocultivation on a chip to observe the cell distribution and interactions at a small scale (different cells can be easily identified in cocultures when an autofluorescent methanogenic archaeal strain (*i.e.*, cofactor F420) and a bacterial strain) are mixed).

**Evolution of deep underground microbial communities.** Omics approaches have been used to gain a more in-depth understanding of the functions of microbial communities in the deep subsurface but have also led to new questions. For example, in the past, any presence of aerobic microorganisms in anoxic deep continental environments was clearly identified as contamination during sampling, which quickly led to a debate. Recently, omics approaches have revealed the presence of active aerobic organisms (Ruff, 2023), even those known only as oxygenic phototrophs (Puente-Sánchez, 2018), which has led to hypotheses on the presence of O<sub>2</sub> in these environments (Winograd, 1982; Gutsalo, 1971; Kadnikov, 2018). Several biogeochemical explanations have been proposed to explain the presence of this electron acceptor, ranging from radiolysis to the dismutation of various molecules (Coates, 2004; Ettwig, 2010; Heck, 2010). In the case of cyanobacteria found in deep continental environments, H<sub>2</sub> consumption via their hydrogenases was hypothesized. Omic approaches are very powerful and informative, but they do not exempt the microbiologist from testing the hypotheses formulated by cultural approaches that clearly demonstrate hypothetical metabolisms and calculate yields to understand the impact on the functioning of the microbial ecosystem. The rapid development of microfluidic tools for isolating and screening prokaryotes in

499 recent years (Yin, 2022; Wan, 2023) raises hopes for the transfer of such technologies to the deep  
500 biosphere community. Indeed, microbial isolation could be carried out directly on water samples  
501 from aquifers or faults and would consume only small volumes (a few microliters) of these often  
502 precious and rare samples.

### 503 **3.4 Current limitations of microfluidic approaches for studying the deep underground** 504 **biosphere**

505 While microfluidics approaches can help address several scientific questions and complement  
506 conventional equipment and strategies to investigate the deep underground biosphere, some  
507 limitations specific to such methodologies exist. We discuss hereafter some of them along with  
508 remediation strategies.

509  
510 **The difficulties in accessing microreactor technologies for new users and accessing adapted HP**  
511 **equipment.** Microreactor technology, especially for polymer microfluidics, has become more  
512 accessible over 15 years (microfabrication kits are now commercially available), although they do  
513 not fully meet deep biosphere research needs. While robust materials such as glass or silicon require  
514 expertise, clean rooms are not always necessary—a fume hood suffices. The complexity of  
515 photolithography and etching may deter newcomers, but robust commercial microfluidic devices are  
516 available, simplifying the process. Several commercial companies now sell robust microfluidics  
517 devices in glass or silicon Pyrex to bypass the need to develop complex and expensive in-house  
518 microfabrication processes along with all the required equipment to proceed in a user-friendly way  
519 with microfluidic studies (Micronit, Little Things Factory, etc.). The overall equipment cost for  
520 performing microbial studies in a microreactor is, of course, slightly greater than that of conventional  
521 microbial incubation methods (with the exception of pressurized cultivation), but such approaches  
522 can (i) yield information that cannot be obtained otherwise (single-cell measurement, bioclogging in  
523 pore structures, etc.) and (ii) greatly reduce the experimental time owing to microfluidic fast-  
524 screening strategies, thus reducing the overall cost in terms of consumables, for example.

525  
526 **Microfluidic anoxic experiments.** Most of the studies dealing with the deep underground biosphere  
527 require anoxic environments to be representative of realistic conditions. As mentioned earlier,  
528 conventional polymer microreactors cannot be considered for such conditions given that oxygen can  
529 diffuse through these materials. Hence, inorganic microfabrication materials are needed, which

530 increases the microfabrication protocol needed unless these materials are ordered from specialized  
531 companies. However, in addition to this limitation, anoxic experiments on a chip are similar to those  
532 on larger-scale reactors. This means that a glove or an intergas ramp box is generally needed to  
533 ensure the elimination of O<sub>2</sub> when the starting culture medium is prepared.

534

535 **The implementation of characterization techniques in microreactors.** While spectroscopy  
536 techniques can be easily implemented on a chip (Raman, FTIR, fluorescence, optical observations,  
537 etc.) and are relatively convenient for studying microbiological processes, they depend on the  
538 microreactor material, particularly in terms of transparency. Therefore, it is important to pay attention  
539 to the choice of construction materials. As mentioned earlier, X-ray analysis and imaging can also be  
540 used in association with microreactors, providing ways to characterize fluid flows and geochemistry  
541 in porous media. Spectroscopy techniques (Raman, FTIR, fluorescence) can be used directly inside  
542 microreactors without disturbing the system. Measurements of gases and metabolites in microscale  
543 volumes have already been reported using confocal Raman spectroscopy to quantitatively monitor  
544 the solubility of gases such as dissolved CO<sub>2</sub> and CH<sub>4</sub>, electrode array detectors or fluorescence  
545 detection (Kraly et al. 2009; Liu et al. 2012). These are powerful approaches for performing direct  
546 biochemical imaging (especially when considering porous media), providing important spatial  
547 information on microbial activities (Loutherback et al. 2016; Marcsisin et al. 2012; Perro et al. 2016).  
548 Additionally, they could also possibly be used for strain identification and quantification on the basis  
549 of specific biomolecular fingerprints without staining. However, compared with conventional larger-  
550 scale volume strategies, semiquantitative analyses are generally still considered. Hence, both  
551 methodologies need to be considered in parallel. *In situ* sequencing is still a tricky problem with  
552 microfluidic methodologies given the small sample volume considered, from picoliter (a drop) to few  
553 microliters (a chip). Hence, microreactors need to be coupled with milliliter-scale reactors to  
554 guarantee sufficient biomass for further analysis. However, recent trends in single-cell RNA/DNA  
555 sequencing (Clark *et al.* 2023; Zhou *et al.* 2021) could lead to the development of online  
556 microfluidics systems in the near future.

557

## 558 **Conclusions and perspectives**

559 While microfluidic tools have revolutionized molecular biology and health care in recent years, the  
560 possibilities of applying these approaches to the study of the deep biosphere have barely scratched  
561 the surface. However, these tools have long been used in the oil industry and in gas storage in the

562 broadest sense. Despite undeniable advantages over large-scale or core-scale methodologies, the  
563 main criticism that can be made to microfluidics is the problem of scaling up the results obtained  
564 with a micromodel to model a reservoir as a whole . For deep subsurface microbiologists, however,  
565 these reservations are not very important in view of the advantages of such approaches, such as (i)  
566 the ability to operate in a large range of experimental conditions (p, T, concentration, etc.),  
567 representative of the deep underground environment; (ii) the safety related to microscale  
568 experimentation; (iii) the possibility of finely tuning the design of the microreactor from very simple  
569 micromodels to the complex permeability and porosity of certain rocks; (iv) the implementation of a  
570 wide range of noninvasive in situ characterization techniques to monitor the growth and behavior of  
571 microorganisms, the composition of fluids, etc.; (v) the possibility of performing numerous replicates  
572 on the same micromodel, making it possible to confirm the results obtained via statistical approaches;  
573 and (vi) the ability to perform fast screening through the utilization of on-chip gradients (T, pH,  
574 concentration, etc.) for the exploration of multiple conditions in a single run and (vii) the isolation of  
575 microorganisms while maintaining pressure conditions and consuming minimal volumes of  
576 formation water.

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577

578 **Conflict of Interest**

579 The authors declare that the research was conducted in the absence of any commercial or financial  
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581 **Author Contributions**

582 AC, SM and ARP designed and funded this research project. SMO, EV, AC, SM and ARP wrote  
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1173 **Table 1:** Comparison of conventional (high-pressure) techniques and microfluidics techniques for  
 1174 the study of deep underground environments.

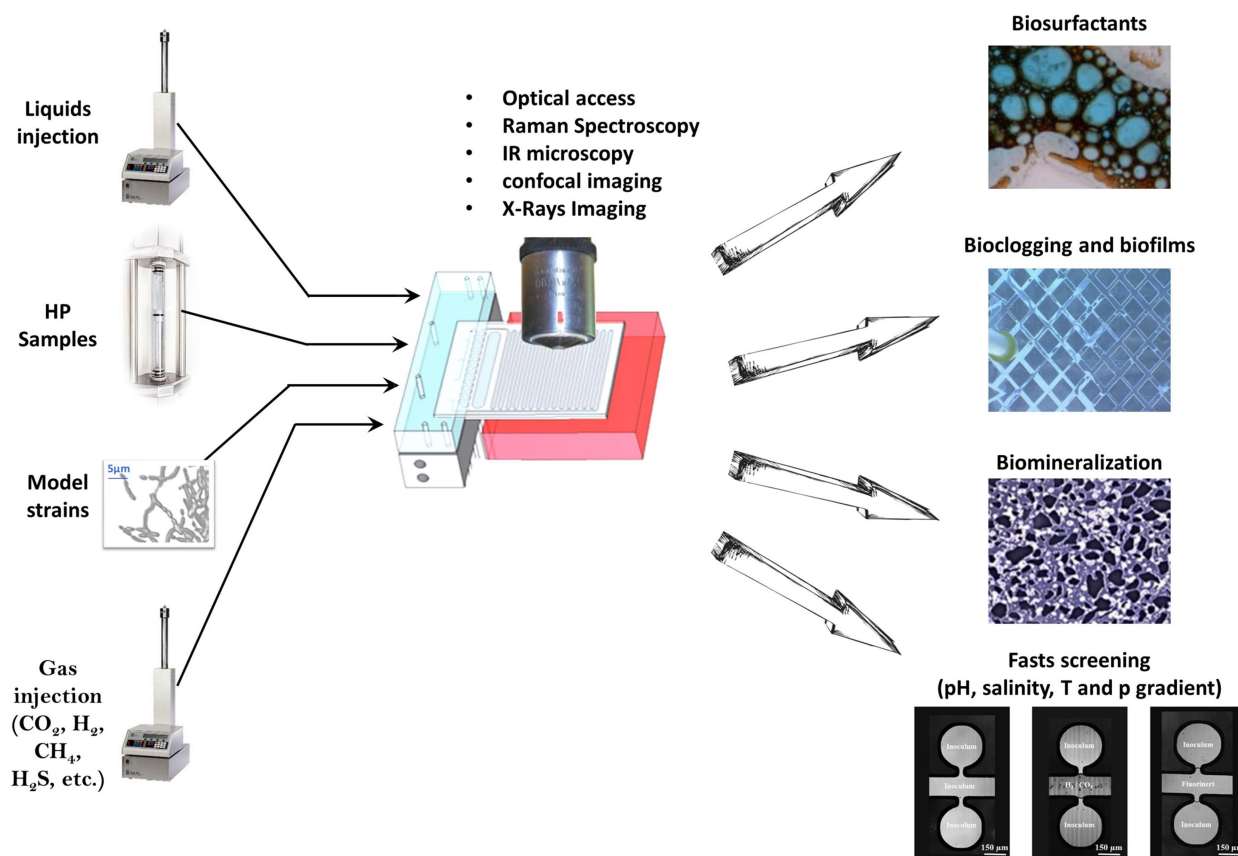
Set-up	Material	Conditions (P, T)	Advantages	Limitations	References
Core-flood	Rock	Up to 40 MPa and 400 °C	Integration of mineralogy, porosity	Nontransparent (except to X- rays)	(Sun, 2011)
Flow- through bioreactor	Hastelloy, PEEK chamber	Pressure up to 20 MPa Temperature up to 120 °C	Continuous monitoring of geochemical and biogeochemical evolution  Wide range of experiments with in situ conditions  Large volumes (mL to L)	Nontransparent	(Dupraz, 2009)
Static pressure vessels	Different alloys (stainless steel, etc.)	Up to 80 MPa and 150 °C	Large volumes  Liquid and solid phases	Requires decompression to subsample  Nontransparent	(Haddad, 2022)
Capillaries	Glass	Up to 100 MPa and 200 °C	Transparent  Precise conditions	Small volume ( $\mu$ L–mL)  Poor design flexibility	(Li, 2014)
Microchips	Silicon substrate	Up to 40 MPa and 400 °C	Pore-scale studies  Precise manipulation of fluids	Small volume (nL to $\mu$ L)	(Lifton, 2016; Morais, 2016)
	Silicon- Pyrex		High throughput screening		

	Rock-based (and polymers)		3D geometry, reactive	Single use	(Park, 2011)
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1179 **Figure 1:** General scheme of the microreactor setup and applications for deep underground  
 1180 biosphere investigations.

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