Iron-oxidizer hotspots formed by intermittent oxic–anoxic fluid mixing in fractured rocks

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Deep iron-oxidizing bacteria sustained by intermittent oxygen delivery through fractures

Olivier Bochet¹, Lorine Bethencourt², Alexis Dufresne², Julien Farasin¹, Mathieu Pedrot¹, Thierry Labasque¹, Eliot Chatton¹, Nicolas Lavenant¹, Christophe Petton¹, Benjamin W. Abbott^{3,2}, Luc Aquilina¹ & Tanguy Le Borgne^{1*}

¹Geosciences Rennes laboratory, UMR 6118 CNRS University of Rennes 1, France

²Ecobio laboratory, UMR 6553 CNRS University of Rennes 1, France

³Brigham Young University, Department of Plant and Wildlife Sciences, Provo, USA

Subsurface environments host most of the freshwater on Earth as well as diverse microorganisms that may constitute a significant part of the biosphere. However, the dynamics and spatial distribution of subsurface microorganisms and their response to hydrological processes are poorly understood. Here we used chemical and metagenomic analysis of groundwater in a fractured rock aquifer in western France to determine the role of fractures in the formation of deep microbial hot spots in the subsurface. The majority of fractures, sampled in a 130-meter-deep borehole, were anoxic, but a fracture carrying oxic groundwater was detected at depths from 37 to 59 meters, associated with a five-fold increase in the abundance of iron-oxidizing bacteria. We developed a mechanistic model of fluid flow and mixing in fractures and found that such microbial hot spots are sustained by the mixing of fluids with contrasting redox chemistries at intersections of fractures. The model predicted that meter-scale changes in near-surface water table levels cause intermittent oxygen delivery through

deep fractures, which can extend the depth of the habitable zone for iron-oxidizing bacteria hundreds of meters into the subsurface. Given that fractures are ubiquitous at multiple scales in the subsurface, such deep microbial hot spots may substantially influence microbial communities and their effect on Earth's biogeochemical cycles.

Water flow transports nutrients and energy for microorganisms, sustaining enhanced reaction rates by delivering missing reactants for fluid-fluid or fluid-mineral reactions¹⁻³. Highly reactive hot spots often form at the intersection of hydrological flow paths, where the confluence of complementary reactants promotes biogeochemical activity that can disproportionately influence larger-scale production rates⁴⁻⁷. These hot spots or ecosystem control points⁸ depend on strong redox and concentration gradients⁹⁻¹¹, and though they change in space and time, they often occur in areas where groundwater mixes with surface water bodies^{5,12,13}. Reactive hot spots occurring near the land or sediment surface can be readily identified, but deep hot spots are difficult to access and generally neglected because most biogeochemical models do not predict their occurrence^{2,14}. Drilling campaigns in the continental crust and in the ocean floor have revealed that deep environments host diverse microbial communities¹⁵⁻²⁰, which support high rates of biogeochemical transformation²¹. However, the spatial patterns of the deep biosphere remain largely unknown and there is currently no mechanistic framework to predict the location and duration of deep microbial hot spots^{22,23}.

A common justification for disregarding deep mixing hot spots is that groundwater flow lines are not expected to intersect in homogeneous subsurface domains (Fig. 1a). In this conventional

view, mixing hot spots only form in groundwater discharge areas where flow lines converge⁵. As water flows along subsurface flow paths, chemical species are generally consumed in the order of their redox potential²¹, leading to vertical stratification of oxidized species depending on water residence time (Fig. 1a). This stable redox gradient could slow down biogeochemical activity and prevent the development of mixing hot spots in the deep subsurface. Here, we tested this longstanding view using quantitative hydrological and microbiological methods. We hypothesized that reactive microbial hot spots can occur over a large range of depths because of the presence of connected fractures at multiple scales in the subsurface^{24,25}. These structural discontinuities produce preferential flow paths, which can mix water with very different residence times and redox properties²⁶. This short circuiting of hydrological flow paths can produce reactive hot spots at much greater depths than predicted for homogeneous environments (Fig. 1b).

Observation of subsurface microbial mats in fractured rock

To test the hypothesis of fracture-induced microbial hot spots in the deep biosphere, we analyzed the biological and chemical properties of fracture fluid in a borehole where a massive microbial mat was formed by iron-oxidizing bacteria (FeOB) dozens of meters below the surface (see Supplementary Movie) in the fractured rock observatory of Ploemeur, France, part of the H+ network of hydrogeological observatories (<http://hplus.ore.fr/en/ploemeur>). FeOB are emblematic of mixing zones, where they take advantage of redox gradients from groundwater inflows rich in reduced iron encountering oxygenated environments²⁷. At circumneutral pH, soluble Fe(II) is readily oxidized by O_2 , forming iron oxyhydroxides which immediately precipitate from solution.

61 Neutrophilic FeOB are only able to outcompete this abiotic oxidation in specific microaerobic
62 conditions ($0.1 < O_2 < 1.5 \text{ mg.L}^{-1}$)²⁸, where they can form thick microbial mats, for instance, in
63 stream beds^{29,30}, intertidal zones³¹, groundwater seeps³², and deep-sea vents³³. As Fe cycling is
64 coupled with the biogeochemical cycles of carbon, nitrogen, and sulfur through both biotic and abi-
65 otic reactions²⁷, FeOB may exert an important control on the biogeochemistry of both continental³⁴
66 and oceanic^{23,35} subsurface environments. In continental systems, neutrophilic, oxygen-dependent
67 iron-oxidizing bacteria are mostly Betaproteobacteria represented by the *Leptothrix* genus³⁰ and
68 members of the Gallionellaceae family: *Gallionella*, *Sideroxydans*, and *Ferriphaselus*^{36,37}. Gal-
69 lionellaceae are thought to be autotrophs that use dissolved CO₂ as a carbon source^{36,37}. In under-
70 ground ecosystems, this primary production could be an important organic carbon source fueling
71 heterotrophic microorganisms. Gallionellaceae appear to be dominant in fractured rocks³⁴, where
72 fluids are rich in dissolved iron, serving as electron donor for FeOB. However, the pathways and
73 dynamics of electron acceptor delivery (e.g. dissolved oxygen or nitrate) in these environments are
74 poorly understood.

75 The studied fractured rock aquifer is partitioned into two chemically distinct zones: recharge
76 areas, located in high topographic positions, where water is oxygen-rich, and discharge areas, lo-
77 cated in low topographic positions, which are poor in oxygen and rich in dissolved iron (see Meth-
78 ods and Extended Data Fig. 1). In recharge areas, downward oxygen fluxes are driven by a positive
79 difference between near surface and deep hydraulic heads. In discharge areas, upward dissolved
80 iron fluxes are driven by negative difference between these hydraulic heads (see Extended Data
81 Fig. 1). Flow primarily occurs in a network of fractures that extends through granite and micas-

chist bedrock (see Methods)^{38,39}. The PZ26 borehole is a 130 m deep artesian borehole located in a discharge area, where a borehole camera survey revealed the presence of a massive rust-colored mat (Fig. 2a, Extended Data Fig. 4, and Supplementary Movie). Borehole optical logs and video images showed that the borehole walls were entirely covered from the surface to approximately a 60 m depth by the rust-colored mat. Growth was particularly profuse where the main permeable fractures intersected the borehole, in places completely clogging the borehole. Below 60 m, only traces of the mat were visible, though there was still evidence of microbial growth at the main fractures. All fractures are under pressure and constantly produce flow towards the top of the borehole (see Methods and Extended Data Fig. 5). Therefore, dissolved oxygen cannot diffuse from the surface through the borehole and any oxic fluid contributing to the observed microbial iron oxidation must flow through the fracture network from the surface down to 60 meters. Along this fracture pathway, the transported oxic fluid is likely to have multiple opportunities to mix with iron rich fluid at intersections with anoxic fractures (Fig. 1b). We combined hydrogeological, geochemical, and metagenomic analysis to investigate the resulting development of deep microbial hot spots of iron oxidizing bacteria within the fracture network. Because mixing of different fracture fluids in the borehole could influence chemical and biological properties, we collected fracture fluid directly from fractures using packers^{40,41} (hydraulic obturators, see Methods and Extended Data Fig. 5), which allow isolating the fractures from the borehole and sampling undisturbed fracture fluid, i.e. not influenced by the borehole.

Fracture distribution and chemical composition

The depth and hydraulic properties of the main permeable fracture were quantified from borehole flowmeter logging and optical borehole logging (see Fig. 2b, Methods and Extended Data Table 1). More than half of the flow in borehole PZ26 was produced by a highly permeable zone at the bottom of the borehole (below 94 m). The rest of the flux came from various shallower fractures, each constituting less than 10 % of the total flow. Estimation of fracture dips and density from optical and acoustic logs (Fig. 2c) indicated that the density of fractures was on average 1.5 fractures per meter in the first 60 meters and close to 1 fracture per meter in the lower part of the borehole. The average fracture dip was about 60 degrees above 60 meters, and about 40 degrees below 60 meters. The zone with the maximum average fracture dip (60 to 80 degrees) was located between 45 and 65 meters (Fig. 2c).

To characterize the properties of fracture fluid and investigate the existence of natural microbial hot spots within the fracture network, we performed vertically-discrete sampling of pristine fracture fluid with an inflatable packer capable of blocking vertical flow (see Methods). We sampled water from each of the main fractures along the entire length of the borehole and analyzed major ions, dissolved gases and microbial diversity (Fig. 2d, Fig. 3 and Methods). The depths of the sampled fractures were known with a good precision from the optical and flow logs (Extended Data Table 1). Based on the volume pumped before sampling ($V = 100L$) and the estimated fracture aperture, the sampling radius ranged from 4 to 8 meters from the borehole (Extended Data Table 1). Hence, combining optical and flow logs with packer sampling allowed precise characterization

of the spatial distribution of chemical properties and microbial communities in the subsurface. Iron concentration generally increased with depth, while oxygen concentration decreased, but there was a significant anomaly of high oxygen concentration (4 mg.L^{-1}) in the F54 fracture intersecting the borehole at 54 m, suggesting a high degree of connectivity of the associated fracture network with the surface (Fig. 2d). This was consistent with the high chlorofluorocarbon (CFC) 113 concentration measured at the same depth, indicating short residence-time⁴², and with the increase of the average fracture dip between 45 and 65 meters, indicating the presence of numerous sub-vertical fractures, which may facilitate connectivity with the surface (Fig. 2c). Ground Penetrating Radar imaging of saline tracer transport in fractures obtained in a nearby borehole indicated the existence of numerous intersections between permeable fractures³⁹. These intersections create high vertical connectivity of the fracture network and act as hydrological mixing zones and biogeochemical hot spots when intersecting fractures carry chemically distinct fluids. The 3D representation of the permeable fractures intersecting the borehole provided a view of the fracture network organization, suggesting that connections between the oxic fracture and anoxic fractures occurred outside of the borehole (Fig. 2e). Comparison of borehole oxygen profiles measured during a recharge period (April 2017) and a dry period (July 2017) showed that oxygen delivery at depth through fractures was intermittent in time (Fig. 2f and Extended Data Fig. 3). During the recharge period, most fractures above 60 meters delivered oxygen at depth, while no oxygen was detected in the borehole during the dry period.

Deep hot spot of iron oxidizing bacteria

Scanning electron microscope observations performed on samples from batch experiments and from the borehole showed typical structures of known iron-oxidizing bacteria (see Methods). The most abundant structures were sheaths (Fig. 3a), which can be formed by *Leptothrix ochracea*, and twisted stalks (Fig. 3b), typical of *Gallionella ferruginea*⁴³ and *Ferriphaselus amnicola*⁴⁴. Batch incubations were performed to characterize the mechanisms of biofilm formation and its effect on water chemistry (see Methods). For these experiments, water was sampled from the top of the borehole, which represents a mixture of water produced from the different fractures in this artesian borehole (all fractures are under pressure and produce water that is constantly flowing out of the borehole). Batch results confirmed that the main reaction occurring when mixing fluid from different fractures was iron oxidation by FeOB. Carbon needed for bacterial growth was provided by dissolved inorganic carbon. Such reactions are expected to occur naturally at intersections of oxic and anoxic fractures (Fig. 1b).

To assess the diversity and the relative abundance of FeOB from water samples collected in fractures, we performed a metagenomic characterization of batch incubation and groundwater-associated communities. Taxonomic classification of metagenomic reads from fluids sampled in fractures showed highly diverse communities with more than 155 phyla and candidate divisions of Bacteria and Archaea (see Supplementary Information). Metagenomes from water enriched in FeOB during batch incubations were further used to reconstruct genomes of the main FeOB from assembly and binning of metagenomic sequences (see Supplementary Information). This

yielded nine draft genomes (i.e. clusters of large genomic fragments or contigs) affiliated with the Gallionellaceae family (see Supplementary Table S1). Phylogenomic analyses based on universal protein-coding genes showed that seven genomes were related to the three Gallionellaceae genera already described. Two additional genomes appeared more distantly related, suggesting that they could belong to a new lineage of undescribed Gallionellaceae (see Supplementary Fig. S4 and Fig. S5). Abundant microtubular sheaths, until now attributed to *Leptothrix ochracea*³⁰, were observed in batch and borehole samples, but the *Leptothrix* genome was not detected in our samples.

The proportion of reads aligning with the Gallionellaceae genomes was variable among fractures ranging from 0.25 % in the fractures located above 30 meters and below 60 meters depth to 1.5 and 1.3 % respectively in fractures F37 and F54/59 (Fig. 3c). This large increase in relative abundance with respect to the background (about 600 % in F37 and 500 % in F54/59) placed FeOB in second position in the ranking of bacteria family in F37 and in first position in F54/F59 (see Fig. S3 in the Supplementary Information). In this context of highly diverse subsurface bacterial communities, the relative abundances of the dominant bacteria in fractures did not exceed one to two percent, as found in other studies using a similar genome-resolved approach⁴⁵. Hence, the two criteria that we use to define the FeOB hot spot are: i) a 500 to 600 % increase of the FeOB relative abundance in fractures F37 and F54/59 relative to the background, ii) a respective second and first position of FeOB in the relative abundance ranking of bacteria families in these two fractures, while they were ranked much lower in other fractures.

The detected microbial hot spot coincided with the location of the oxic fracture zone that

delivered oxygen from the surface to depth through a network of mostly anoxic fractures, rich in dissolved iron (Fig. 2). Therefore the origin of this hot spot was likely the intersection oxic and anoxic fractures that created locally favorable conditions for microbial iron oxidation in the fracture network (Fig. 1b and 2f). Genes associated with denitrification pathways were detected in some of the assembled *Galionellaceae* genomes (data not shown). However, measured nitrate concentration was always below the detection limit, suggesting that oxygen was the main electron acceptor for iron oxidation. Note that the continuous presence of a dense microbial mat in the borehole from 60 meters to the top of the borehole was likely due to the upflow of fracture fluid from fractures F37 and F54/F59 to the surface because of the artesian nature of the borehole. This allowed for the colonization of the upper part borehole by FeOB, which are known to create large amounts of mat. Because iron oxidation does not yield much energy, FeOB need to oxidize a large quantity of reduced iron to sustain their metabolism^{27,29}.

Fracture fluid mixing and intermittent oxygen delivery

We combined flow, fracture, and oxygen logs with chemical composition of fracture fluids in the borehole to develop a mechanistic model explaining and predicting quantitatively the formation of deep microbial hot spots in the subsurface. During recharge periods, sub-vertical fractures act as preferential flow paths to transport oxygen-rich water from the surface to the deep subsurface. Because such transport occurs through a multiscale fracture network composed of fractures of broadly different lengths and orientations⁴⁶, there are multiple opportunities for mixing with old groundwater that is rich in dissolved mineral species at intersections of oxic and anoxic fractures.

This effectively creates a short-circuiting of the stratified redox sequence expected for homogeneous media (Fig. 1a). Iron-oxidizing microbial communities can then develop at intersections between oxygenated and anoxic fractures at much greater depths than expected in the conventional view (Fig. 1b). Seasonal fluctuations in recharge (Extended Data Fig. 3a) generate intermittent oxygen delivery, likely inducing temporally dynamic microbial growth (Fig. 2f). The proposed mechanism of fracture-induced mixing may be particularly significant in the fractured rocks that constitute continental aquifers^{21,34} as well as in the oceanic crust where other taxa of iron-oxidizing bacteria are present¹⁸. Our model predicts the range of depths where microaerobic conditions necessary for FeOB are met⁴⁷ by quantifying change in oxygen due to biogeochemical reactions along the fracture flow path and mixing at fracture intersections.

We considered an intersection between two fractures that connected oxidized and reduced flow paths (Fig. 1c), characterized respectively by the hydraulic transmissivities T_{ox} and T_{red} and the oxygen concentrations $O_2^{ox} \gg O_2^{red}$. We assumed that water flow in the oxidized fracture Q_{ox} was driven by the local hydraulic head difference between the shallow and deep subsurface dh (Fig. 1b), and that flow in the reduced fracture Q_{red} was driven by a regional hydraulic head gradient ∇h_r (Fig. 1c). Furthermore, we assumed that oxygen consumption followed a first order kinetic when transported by fracture flow. The oxygen concentration at fracture intersections was then calculated assuming complete mixing at fracture intersections from a flux balance for different depths and hydrological parameters (see Methods and Supplementary Information). Baseline values for all parameters were estimated from observed conditions at the field site (Table 1).

As hydraulic transmissivity values can span orders of magnitude in fractured media^{24,38}, we tested the sensitivity to this parameter of the location of the microbial oxidation zone (defined as the range of depths where $0.1 < O_2 < 1.5 \text{ mg.L}^{-1}$)²⁸ (Fig. 4a), holding all other factors constant. For low transmissivity ratios $T_{ox}/T_{red} < 10^{-3}$, the microbial oxidation zone was constrained to shallow depths, but for intermediate ratios $10^{-2} < T_{ox}/T_{red} < 10^{-1}$, the reactive zone occurred over a large range of depths (Fig. 4a). At larger ratios, chemical oxidation became dominant. When model parameters were set to match observed conditions at the field site (Table 1), the transmissivity ratio was 10^{-2} , which corresponds to the transition between shallow and deep microbial oxidation regimes. In this situation, the depths favorable for FeOB development ranged from approximately 450 to 40 meters, which includes the depth of the observed microbial mat formation (60 meters). While current representations dissociate surface, oxygen-rich ecosystems from reduced, deep ecosystem, this result reveals the existence of a thick reactive zone where mixed conditions can occur at fracture intersections, potentially contributing to recycling chemical species originating from rock weathering²¹.

Because the flux of water and associated dissolved oxygen is proportional to the hydraulic head difference dh between the near-surface and deep compartments, the range of depths favorable for FeOB development is expected to vary seasonally (Fig. 4b), following the intermittent oxygen delivery by fractures. At the field site, the near-surface hydraulic head varies between the recharge period and the dry season, and the deep hydraulic head is relatively stable (Extended Data Fig. 3a). According to model predictions (Fig. 4b), the resulting meter-scale fluctuations of the hydraulic head difference between the shallow and deep compartments dh can generate hundred-meter-scale

fluctuations of the maximum depth of the FeOB habitable zone. This is consistent with observed large changes in oxygen fluxes through permeable fractures observed between the recharge and dry seasons at our field site (Fig. 2f and Extended Data Fig. 3b). This result demonstrates that the depth of the microbial oxidation zone is highly sensitive to fluctuations in recharge at the surface, which challenges the general view that deep subsurface environments respond slowly and weakly to surface changes.

The presented model provides a mechanistic explanation for the observed deep microbial hot spots and allows exploration of the sensitivity of this phenomenon to hydrological flow path structure and temporal dynamics. This quantitative framework could be used to assess the likelihood of occurrence of different types of biogeochemical reactions besides iron-oxidation and their consequences for the transport and transformation of chemical elements, nutrients, and contaminants in hydrological systems^{21,22,27}, particularly in the context of critical zone studies that investigate coupled processes in the aquifer-catchment-atmosphere continuum². While the deep subsurface is usually considered as biogeochemically buffered³⁴, because of its supposed isolation from surface hydrological fluctuations, our results suggest that subterranean biogeochemistry may be spatially heterogeneous and temporally dynamic. Favorable conditions for reactive hot spot development are likely seasonal, creating hot moments of microbial activity at different depths and different times of the year. Because the subsurface represents considerable volumes of water and fluxes of dissolved elements⁴⁸, the identification of fracture-induced hot spots could influence landscape-scale biogeochemical cycles²¹. More generally, the presence of large and dynamic subsurface microbial communities has fundamental implications for our understanding of the location

and timing of biogeochemical activity in the Earth's ecosystems¹⁴. The existence of fractures at multiple scales in the Earth's subsurface²⁴ suggests that the mechanisms described here may be pervasive and potentially involve a large range of biogeochemical processes, driven by mixing of fluids with different residence times and chemical compositions¹⁰.

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Corresponding Author

Correspondence and requests for materials should be addressed to Tanguy Le Borgne, tanguy.leborgne@univ-rennes1.fr

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Author Contributions

O.B. lead the field campaigns, batch tests, data interpretation, model development and results formatting, L.B. carried out metagenomic analysis, A.D. supervised metagenomic data interpretation, J.F. managed bacteria sampling and characterization in field campaigns and batch experiments, and

Table 1: Baseline parameters for the fracture mixing model estimated from field data (see Supplementary Information).

Parameter	Estimated value	Parameter	Estimated value
z	50 m	T_{ox}	$10^{-5} \text{ m}^2.\text{s}^{-1}$
α	60°	z_c	850 m
∇h_r	3.10^{-3}	z_d	23 m
dh	8 m	O_2^0	4.5 mg.L^{-1}
T_{red}	$10^{-3} \text{ m}^2.\text{s}^{-1}$	O_2^{min}	0.1 mg.L^{-1}

performed hydrochemical borehole logging, M.P. carried out chemical analysis in field campaigns and batch experiments, T.L. managed measurements of dissolved gases and CFC in field campaigns, N.L. developed the packer system and managed borehole flow and pressure measurements, C.P. handled logistics related to field campaigns, B.W.A. contributed to formalize biogeochemical implications of results, manuscript editing and proofreading, L.A. supervised geochemical and metagenomic data interpretation, T.L.B. designed the research and supervised data interpretation, modelling and manuscript writing.