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# Production of Current by Syntrophy Between Exoelectrogenic and Fermentative Hyperthermophilic Microorganisms in Heterotrophic Biofilm from a Deep-Sea Hydrothermal Chimney

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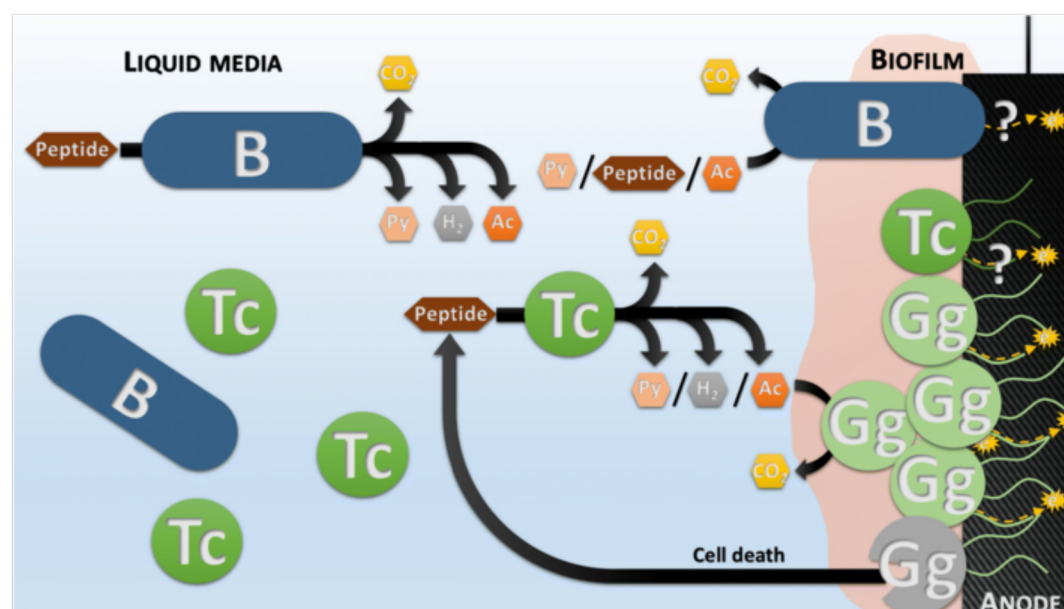
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## Abstract

To study the role of exoelectrogens within the trophic network of deep-sea hydrothermal vents, we performed successive subcultures of a hyperthermophilic community from a hydrothermal chimney sample on a mix of electron donors in a microbial fuel cell system. Electrode (the electron acceptor) was swapped every week to enable fresh development from spent media as inoculum. The MFC at 80 °C yielded maximum current production increasing from 159 to 247 mA m<sup>-2</sup> over the subcultures. The experiments demonstrated direct production of electric current from acetate, pyruvate, and H<sub>2</sub> and indirect production from yeast extract and peptone through the production of H<sub>2</sub> and acetate from fermentation. The microorganisms found in on-electrode communities were mainly affiliated to exoelectrogenic *Archaeoglobales* and *Thermococcales* species, whereas in liquid media, the communities were mainly affiliated to fermentative *Bacillales* and *Thermococcales* species. The work shows interactions between fermentative microorganisms degrading complex organic matter into fermentation products that are then used by exoelectrogenic microorganisms oxidizing these reduced compounds while respiring on a conductive support. The results confirmed that with carbon cycling, the syntrophic relations between fermentative microorganisms and exoelectrogens could enable some microbes to survive as biofilm in extremely unstable conditions.

## Graphical Abstract

Schematic representation of cross-feeding between fermentative and exoelectrogenic microbes on the surface of the conductive support. B, *Bacillus/Geobacillus* spp.; Tc, *Thermococcales*; Gg, *Geoglobus* spp.; Py, pyruvate; Ac, acetate.



## Keywords

Electromicrobiology, Exoelectrogenic, hyperthermophilic microorganisms, microbial interaction, Deep-sea hydrothermal vent. ~~Note: This data is mandatory. Please provide~~

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## Electronic supplementary material

The online version of this article ( <https://doi.org/10.1007/s00248-019-01381-z> ) contains supplementary material, which is available to authorized users.

## Introduction

The deep-sea hydrothermal vents located along the mid-ocean ridges of the Atlantic, Pacific, and Indian oceans [1, 2] are dynamic habitats characterized by large steep thermal and chemical gradients. Around the hydrothermal chimneys, these gradients provide a wide range of growth conditions for the many extremophilic micro- and macro-organisms composing these specific deep-sea ecosystems [3, 4]. The origin of these ecosystems is thought to be based on (hyper) thermophilic chemolithoautotrophs that use chemical energy from the oxidization of reduced chemical substances to convert inorganic carbon into organic carbon. These organics then serve as primary nutrients for the whole ecosystem [5, 6], enabling the development of heterotrophic microorganisms and macro-organisms through the food web. Studies on the microflora of deep-sea hydrothermal vents have identified hyperthermophilic autotrophs and heterotrophs of both the *Bacteria* and *Archaea* domains in the chimney structures [7]. These autotrophic and heterotrophic microorganisms use a wide diversity of metabolisms based on different electron donors ( $H_2$ ,  $H_2S$ ,  $CH_4$ ,  $Fe^0$ ,  $Fe^{2+}$ ,  $S^0$ , organic matter) and acceptors ( $O_2$ ,  $NO_3^-$ ,  $SO_4^{2-}$ ,  $CO_2$ ,  $Fe^{3+}$ ) found in the environment or produced by other microorganisms [8]. These diverse metabolisms support a complex food web and lend the biocenosis stability in response to critical changes in conditions.

Recent studies on samples from deep-sea hydrothermal vents have revealed a new type of metabolism based on microorganisms called exoelectrogens, which are able to transfer electrons directly from cells to an electrically conductive support like the chimney walls [9, 10, 11].

A decade of research has shown the possibility of electron transfer through the electrically conductive chimney composed of polymetallic massive sulfide. The electric current was not only produced by chemical reaction between reduced compounds in the hydrothermal fluid and the oxidative conditions surrounding the chimney [12], but could also be generated from biological catalysis by exoelectrogenic microorganisms [10]. Thus, a proportion of the hydrothermal vent chimney's microbial populations is likely dependent on energetic metabolism based on these extracellular electron transfers (EET) to insoluble extracellular minerals. Most of what we know about EET in microbes comes from studies using bioelectrochemical systems such as microbial fuel cells (MFCs) [13, 14, 15]. A MFC is a bioelectrochemical device that supports microorganism growth on an anodic electrode, where the microorganisms oxidize substrates used as energy source and transfer electrons to the anode which serves as electron acceptor. The electron flux is then routed toward the cathodic electrode where the reduction of the terminal electron acceptor, usually the oxygen, takes place. In the past few years, exoelectrogenic activity has been reported in almost 100 mesophilic microbial species, mainly affiliated to the bacterial phylum *Proteobacteria* [16]. Studies have shown electric current production by in situ benthic MFCs on hydrothermal systems, with in one case the dominant presence of mesophilic *Bacteroidetes*, *Firmicutes*, and  $\delta$ -*Proteobacteria* [17, 18, 19]. Just recently, three hyperthermophilic archaea, *Pyrococcus furiosus*, *Geoglobus ahangari*, and *Ferroglobus placidus*, were shown to be capable of producing electricity in MFCs [9, 11]. These microorganisms belonging to the orders *Archaeoglobales* and *Thermococcales* have been found in hydrothermal chimneys where they could use a conductive support like the pyrite composing the chimney wall [20] as electron acceptor for their metabolism. Another recent study showed the dominant presence of *Archaeoglobales* and *Thermococcales* in a hyperthermophilic exoelectrogenic community from a deep hydrothermal vent sample enriched using acetate as electron donor in a microbial electrolysis cell (MEC) system with the anode poised at  $-0.110$  V vs Ag/AgCl [10]. While only few electrogenic species should be able to grow under these very selective physicochemical conditions, it is reasonable to think the deep-sea hydrothermal ecosystem harbors a broader exoelectrogenic biodiversity.

The importance of exoelectrogens in the biosphere of hydrothermal ecosystems and their roles within the food web are still unclear. There have been attempts to investigate trophic relationships among the different species inhabiting these environments [21, 22], but most studies to date have focused on relations between microflora and macrofauna, and none have integrated exoelectrogenic ability as a potential metabolism and electron sink. Here, we set out to gain further insight into the role of hyperthermophilic exoelectrogens in the hydrothermal biosphere. An exoelectrogenic community sampled from a deep-sea hydrothermal vent was grown using a hyperthermophilic MFC system. The system was configured with a free-potential anode as sole electron acceptor, and a broad diversity of substrates was tested to enrich an exoelectrogenic community. In order to select a community including both exoelectrogens and other closely related species, the community was grown over 1 month on an anodic support that was replaced each week. Current production was monitored in real time, and changes in biodiversity of the on-electrode biofilm and free cells in the liquid media were determined at each electrode change. During the fourth week, when current density was stabilized, the different substrates (acetate, formate, pyruvate, hydrogen, glycogen, starch, yeast extract, peptone, and without substrate) were separately tested alone to determine the current density production attributable to each one. The results obtained here, along with knowledge on the metabolisms of each species retrieved on both electrode and liquid media, argue for interspecies interactions inside the community, prompting us to propose a putative syntrophic model.

## Materials and Methods

### Sample Collection and Preparation

A hydrothermal chimney sample was collected on the Capelinhos site on the Lucky Strike hydrothermal field (37°17.0' N, MAR) by the remotely operated underwater vehicle VICTOR 6000 during the MoMARSat cruise in 2014 (doi: <https://doi.org/10.17600/14000300>) led by IFREMER (France) on board R/V *Pourquoi Pas?* [23]. The sample (PL583-8) was collected by breaking off a piece of a high-temperature active black smoker using the submersible's robotic arm and bringing it back to the surface in a decontaminated insulated box. On board, chimney fragments were anaerobically crushed in an anaerobic chamber under H<sub>2</sub>:N<sub>2</sub> atmosphere (2.5:97.5) (La Calhene, France), placed in flasks under anaerobic conditions (anoxic seawater at pH 7 with 0.5 mg L<sup>-1</sup> of Na<sub>2</sub>S and N<sub>2</sub>:H<sub>2</sub>:CO<sub>2</sub> (90:5:5) gas atmosphere), and stored at 4 °C. Prior to the experiment, pieces of hydrothermal chimney were removed from the sulfidic seawater flask, crushed with a sterile mortar and pestle in an anaerobic chamber (Coy Laboratories, Grass Lake, Mich.), and distributed into anaerobic tubes for use in the various experiments.

### Exoelectrogenic Enrichment in a Bioelectrochemical System

A bioelectrochemical system (BES) was filled with 1.5 L of sterile mineral medium as previously described [10, 24], and set at 80 °C (monitored by an internal temperature probe). To ensure anaerobic condition, the culture medium was continually sparged with a 50-mL/min flow of N<sub>2</sub>. The pH was maintained at 6.5 ± 0.1 by the addition of sodium hydroxide (NaOH 0.5 mM) or hydrochloric acid (HCl 0.5 mM). The stirring, driven by two axial impellers, was set to 300 rpm. All the parameters, as pH and ORP (Mettler Toledo InPro 3253, Switzerland), temperature (Prosensor pt. 100, France), stirring speed, gaseous H<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub>, O<sub>2</sub>, and CO<sub>2</sub> concentration and NaOH and HCl consumption, were measured and managed by the BatchPro software (Decobecq Automatismes, France). The liquid media were supplemented with 10-mM acetate, 10-mM formate, 10-mM pyruvate, 0.5-g L<sup>-1</sup> yeast extract, 0.5-g L<sup>-1</sup> peptone, 0.5-g L<sup>-1</sup> starch, and 0.1-g L<sup>-1</sup> glycogen as substrate. These compounds, added in excess, can be found in hydrothermal systems through Fischer–Tropsch process reactions, produced by microorganisms for energy storage or released during cell degradation. The working electrode (WE) composed of 20 cm<sup>2</sup> of carbon cloth (PaxiTech SAS, France) served as a potential electron acceptor for exoelectrogen growth. An anion exchange membrane (Membrane International Inc., Ringwood, NJ) separated the two chambers. The counter electrode (CE) was a 20-cm<sup>2</sup> carbon cloth coated with platinum. The system was inoculated with 5 g of crushed chimney in anaerobic conditions. Current production was monitored using SP-240 potentiostats and EC-Lab software (BioLogic, France) via the constant load discharge method, with titanium wire connected to the WE and CE and to a 3M Ag/AgCl reference electrode (Mettler Toledo InPro 3253, Switzerland). Current density and WE and CE potential were measured every 10 s. The resistance imposed between the WE and CE was set at 700 Ω, based on the maximum power density obtained on the polarization curve (see Supplementary Fig. 1), and the system's internal resistance of 675 Ω. Gas production during experiment was monitored every 3 min as previously described [24], using a micro-GC

equipped with a catharometric detector (MS5A, SRA Instrument, France) and a CARBOCAP CO<sub>2</sub> probe (Vaisala GMT 221, Finland) with a sensibility threshold at 0.001%. The WE was swapped three times for a new sterile one, once maximum current density had stabilized for a few days, adding 1250 mL of fresh mineral medium supplemented with substrates (see Supplementary Material & Methods). A volume of 250 mL of spent media from the previous experiment with the environmental inoculum (crushed chimney) was left in the bottom of the reactor before starting each subculture. After the first week, an open-circuit control was performed in a similar way as for week 2 enrichment but with the electrodes disconnected from the potentiostat. Tests on substrates were carried out successively by emptying the spent medium and filling with fresh medium supplemented with a single substrate in the same concentrations as described above. Tests on H<sub>2</sub> were carried out at 50 mL min<sup>-1</sup> of H<sub>2</sub> flux sparged in the agitated bioreactor, which is far higher than observed by fermentation in order to overcome the lack of information on the solubility of our H<sub>2</sub> gas supply compared with the solubility of H<sub>2</sub> produced by fermentation in close vicinity to the electrode. An abiotic control with H<sub>2</sub> was performed in rich liquid media, but no current production was observed. A spent media control was performed in sterile BES filled with 0.2- $\mu$ m filtered culture media from the end of first culture. A killed inoculum control was performed in same conditions as the enrichment experiment but inoculated with chimney sample sterilized at 121 °C during 20 min. Cyclic voltammeteries were carried out at initial time and prior to collecting the WE at 20 mV s<sup>-1</sup> between -0.5 and 0.5 V vs the Ag/AgCl reference electrode. Due to the limited inoculum quantity and accessibility and its quick degradation when opened, even in anaerobic conditions, the experiment could not be replicated.

## Biodiversity Analysis

Taxonomic affiliation was performed according to [25]). DNA was extracted from 1 g of the crushed chimney and at the end of each week of culture from scrapings of half of the WE and from centrifuged pellets of 50 mL of spent media. DNA extraction was carried out using the [PowerSoil DNA Isolation Kit \(MoBio laboratories, Inc., Carlsbad, CA\)](#) ~~MoBio PowerSoil DNA isolation kit (Carlsbad, CA)~~. The V4 region of the 16S rRNA gene was amplified using the universal primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CNN GGG TAT CTA AT-3') [26] with Taq-&Load PCR MasterMix (Promega, Madison, WI). PCR reactions were carried out using a C1000 Thermal Cycler (BioRad) in the following conditions: initial denaturation at 94 °C for 3 min followed by 30 denaturation cycles at 94 °C for 30 s, primer annealing at 50 °C for 30 s and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min. The amplified gene regions were sequenced on an Illumina MiSeq 2500 platform (GeT-PlaGe, France) to generate paired-end 150-bp reads. Reads were merged using FLASH software (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>). Taxonomic affiliation was done using the QIIME software package (v 1.9.1, <http://qiime.org/index.html>). Chimera were removed from the merged sequence using the UCHIME algorithm ([http://www.drive5.com/usearch/manual/uchime\\_algo.html](http://www.drive5.com/usearch/manual/uchime_algo.html)). The filtered sequences were then clustered into OTUs using the RDP method with a minimum bootstrap confidence of 0.8, and OTUs were affiliated using the Silva database as reference (<http://www.arb-silva.de>). To analyze alpha diversity, the OTU tables were rarefied to a sampling depth of 13,190 sequences per library, and two metrics were calculated: the richness component represented by number of OTUs observed, and the evenness index (Pielou's index) which measures distribution of individuals within species independently of species richness. Rarefaction curves (see Supplementary Fig. 2) for each enrichment approached an asymptote, suggesting that the sampling depths were sufficient to capture overall microbial diversities in the studied samples. Week 4 biodiversity was not studied due to the bias of further analysis on substrates which would have specifically influenced the exoelectrogenic community, so we cannot compare week 4 biodiversity to weeks 1–3. The raw sequences for all samples are registered in the European Nucleotide Archive (accession number: PRJEB25397).

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## Quantitative PCR of Archaeal and Bacterial 16S rRNA Gene Copies

Bacterial and archaeal quantification was carried out by qPCR with SsoAdvanced™ Sybr Green Supermix on a CFX96 Real-Time System (C1000 Thermal Cycler, Bio-Rad Laboratories, CA) with the primers GML5F (5'-GCC TAC GGG AGG CAG CAG-3') and Univ516 (5'-GTD TTA CCG CGG CKG CTG RCA-3') specific to *Bacteria* [27] and Arc931F (5'-AGG AAT TGG CGG GGG AGC A-3') and m1100R (5'-BTG GGT CTC GCT CGT TRC C-3') for *Archaea* [28]. The PCR program was composed of a 10-s denaturation step at 94 °C, a hybridization step of 10 s at 55 °C (*Bacteria*) or 62 °C (*Archaea*), and a 10-s elongation step at 72 °C, with

melting curves generated at the end of each reaction to ensure product specificity. A standard curve from  $10^2$  to  $10^{10}$  16S rRNA gene copies was obtained by diluting pGEM-T plasmids harboring hyperthermophilic bacterial or archaeal rRNA gene fragments obtained from the microbial community of interest. Results were expressed in number 16S rRNA gene copies per gram of crushed chimney per milliliter of liquid media or per square centimeter of electrode surface.

## Microscopy Observation with Fluorescent In Situ Hybridization

Prior to microscopic observation, the WE and the liquid media from the end of the week 2 experiment were fixed with 2% paraformaldehyde and kept at 4 °C. The liquid media (100 mL) were then filtrated on a 0.2- $\mu$ m polycarbonate Whatman Nuclepore membrane. To highlight the presence of microorganisms on the electrode surface, Syto9 was used as total fluorescent nucleic acid stain. FISH probes specific to *Bacteria* (EUB338-FITC), *Archaea* (ARCH917-CY3), *Thermococcales* (Tcoc164-FITC), and *Archeoglobales* (Arglo32-FITC) [29] were used separately on different areas of the electrode. Total cell staining was performed by immersing a patch of electrode in 10  $\mu$ M of Syto9 for 15 min then rinsing it several times in sterile water. For FISH observation, samples of WE or cells on filter were incubated with fluorescent probes in an equilibrated humidity chamber at 48 °C for 2–6 h and then washed with washing buffer at 42 °C for 15 min. Samples were then dried in 80% ethanol and mounted on glass slides with antifadent AF1 (Citifluor, Hatfield, PA) added with 4'-6'-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) as counterstain at a final concentration of 2  $\mu$ g mL<sup>-1</sup>. Electrode samples were observed under a confocal LSM780 microscope (ZEISS, Germany) equipped with a  $\times 10$  EC PLAN-NEOFLUAR objective. Blue and green fluorescence emissions were acquired by laser excitation at 358 and 488 nm, respectively. Image stacks (at 0.5–1  $\mu$ m steps) were acquired with GaAsP photomultiplier tube detectors. Epifluorescence micrographs were processed using Zen software (ZEISS, Germany) and Fiji software (<http://imagej.nih.gov/>; <http://fiji.sc/Fiji>). Liquid samples on filters were observed on an Olympus BX61 microscope with a Hamamatsu digital camera c11440, and processed with an Olympus cellSens™ software.

## Results

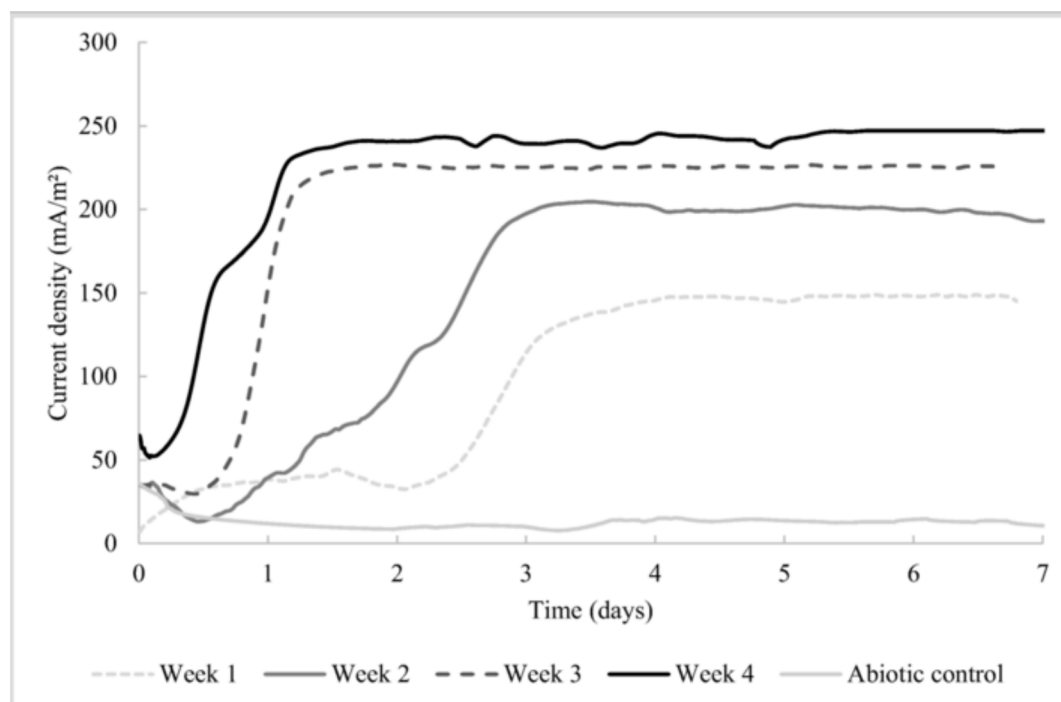
### Exoelectrogenic Community Enrichments and Current Density Production

To allow the growth of a hyperthermophilic exoelectrogenic biofilm (HEB) with a maximum of biodiversity and trophic relations, a MFC was filled with synthetic seawater supplemented with a mix of different carbon and energy sources (acetate, formate, pyruvate, yeast extract, peptone, glycogen, and starch). Current density production was measured using the constant load discharge (CLD) method with a fixed external resistance (700  $\Omega$ ) in the circuit between anode and cathode. Once current stabilization was reached after a few days, the anode was replaced by a sterile one to allow a new development of exoelectrogenic community. Four successive enrichments were done during 4 weeks in order to stabilize the exoelectrogenic community over the subcultures.

The current density production results are reported in Fig. 1 and Table 1. Over the subcultures, current densities increased from 159 to 247 mA m<sup>-2</sup>. This was correlated to an increase of electrical power density ( $P_{\max}$  from 49 to 110 mW m<sup>-2</sup>) while lag time before current production decreased from 52 h (week 1) to 5 h (week 4) (Table 1). These current density and maximum power density values are in good agreement with another study on (hyper) thermophilic MFCs operated at temperature between 55 to 95 °C in similar carbon/energy-source conditions, which found current density ranging from 100 to 350 mA m<sup>-2</sup> and maximum power density ranging from 37 to 165 mW m<sup>-2</sup> [30, 31]. The increase of maximum current density coupled to the decrease of lag phase before current production over each subculture suggests a specialization of the community colonizing the electrode used as an electron acceptor. The increase in current was concomitant with a decrease in H<sub>2</sub> and CO<sub>2</sub> production down to 0.08%(v/v) for both in the MFC gas outlet (100 mL min<sup>-1</sup>), which is typical of fermentative metabolism occurring in the liquid media. In contrast, the abiotic, spent media, and inoculum-killed controls produced a weak current (30–40 mA m<sup>-2</sup>) during the first few hours which rapidly decreased to nearly 5–10 mA m<sup>-2</sup> with no gas production, whereas the open-circuit control showed higher production of H<sub>2</sub> and CO<sub>2</sub> up to 0.2% (v/v) in the gas outlet (see Supplementary Fig. 3).

#### Fig. 1

Current production plotted per week of culture for inoculated experiments (weeks 1, 2, 3, and 4) and abiotic control. Constant load discharge was used with a fixed external resistance set at 700  $\Omega$

**Table 1**

Lag time before current increase, maximum current density  $J_{\max}$  ( $\text{mA m}^{-2}$ ), maximum difference of potential between anode and cathode  $U_{\max}$  (V), and maximum power density  $P_{\max}$  ( $\text{mW m}^{-2}$ ) obtained after current stabilization over 4 weeks of culture

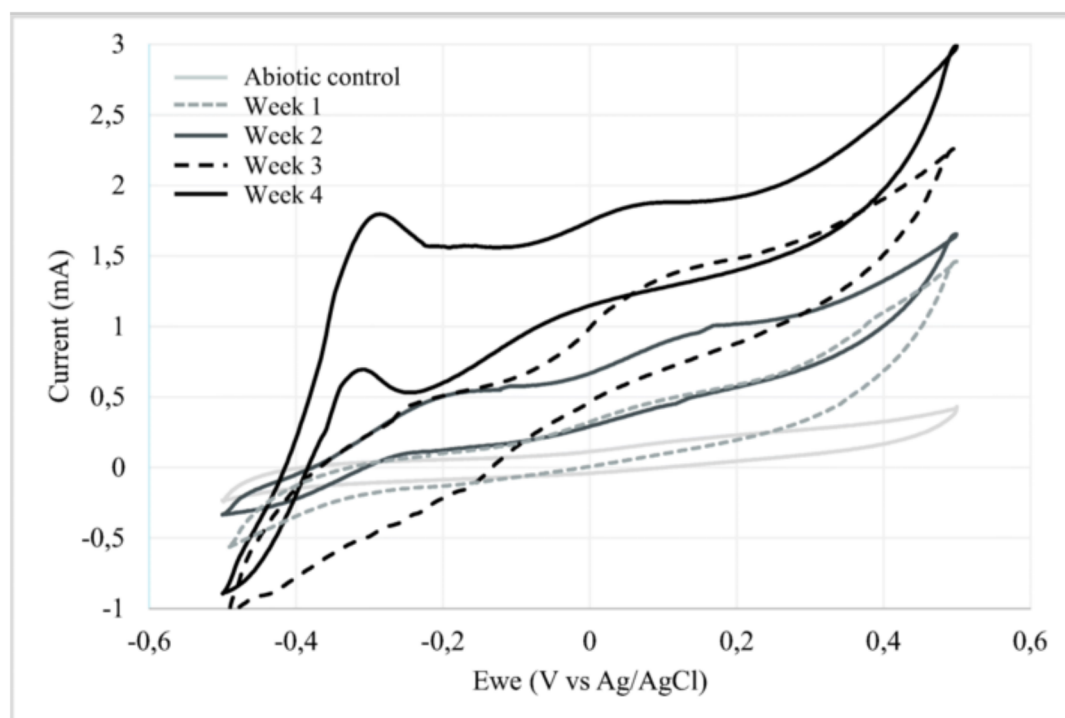
Time	Lag time (h)	$J_{\max}$ ( $\text{mA/m}^2$ )	$U_{\max}$ (V)	$P_{\max}$ ( $\text{mW/m}^2$ )
Week 1	52 h	159	0.29	49
Week 2	19 h	208	0.42	76
Week 3	12 h	225	0.43	90
Week 4	5 h	247	0.46	110

To determine the potential of the oxidation and reduction reactions by the exoelectrogenic biofilm, cyclic voltammetry measurements were performed at the end of each subculture when current density had stabilized. Figure 2 shows the increase in catalytic and capacitive currents after each subculture corresponding to a change in electrochemical properties of the electrode surface that may indicate the development of a HEB. The catalytic current was higher and more distinct than for abiotic controls, suggesting that the current was not caused by the complex medium. The week 4 voltammogram of the MFC anode showed two catalytic oxidation waves: (i) the first started at  $-0.5$  V and increased sharply around  $1.8$  mA at a midpoint potential of about  $-0.360$  V; (ii) the second started at  $-0.18$  V at a midpoint potential of  $-0.014$  V. These oxidation waves increased over the weeks of culture and correspond to the oxidation of different compounds that may be catalyzed by the electroactive biofilm. Similar midpoint potentials at  $-0.350$  V vs. Ag/AgCl electrode have been observed in previous investigations of thermophilic exoelectrogenic enrichments on acetate [11, 32]. A pattern difference was observed in a previous study in MEC inoculated with hydrothermal chimney (Pillot et al., 2018) showing less capacitive current and lower maximum current peaks at  $-0.300$  V. This difference is probably due to the use of only acetate as electron donor, the method of enrichment used with electrode poised at higher potential than observed in this study, and the absence of renewal of the conductive support for exoelectrogenic community selection.

**Fig. 2**

Cyclic voltammetry analysis of the anode per week of culture at  $20 \text{ mV s}^{-1}$  ranged  $-0.5$  to  $0.5$  V. The experiment was performed in triplicate. The curve shows data from a representative experiment





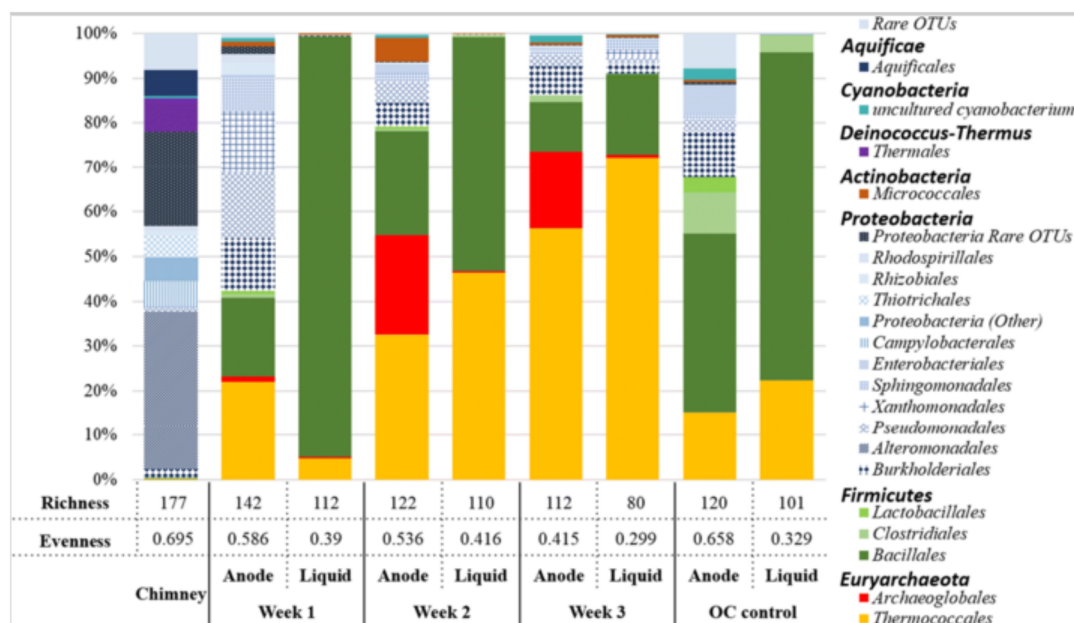
### Evolution in Microbial Diversity During the First 3 Weeks of Enrichment

To identify the evolution of microbial diversity on the electrode and in liquid media over the first 3 weeks of enrichment, a 16S rRNA gene survey was performed by the Illumina MiSeq sequencing the hypervariable V4 region (Fig. 3) of the chimney sample and after each stabilization of current density (at about day 7 for each). During the first 3 weeks of enrichment, biodiversity became poorer with a loss of richness from 177 OTUs in the inoculum to 80 in week 3, correlated to a decrease in evenness index (from 0.695 to 0.299, respectively). This decrease of biodiversity and dominance of a few species can be explained by our selective enrichment conditions with high temperature, substrate specificity, anode electron acceptor, and quick renewal of electrically conductive support.

#### Fig. 3

Dominant taxonomic affiliation at order-level and biodiversity indices of microbial communities from a crushed chimney sample, as plotted on the anode and liquid media over the weeks of culture and in the non-polarized electrode control. OTUs representing less than 1% of total sequences of the samples are pooled as “rare OTUs”. Biodiversity indexes of species richness and evenness represent the number of observed OTUs and Pielou’s evenness ( $J$ ) index, respectively

AQ4



Based on average abundance analysis, the taxonomic composition of the environmental sample (Fig. 3) was dominated by *Bacteria* (99.6% of microbial biodiversity), where *Proteobacteria* (77%) was the major phylum with 50%  $\gamma$ -proteobacteria, mainly represented by *Alteromonadales*, 11%  $\alpha$ -proteobacteria, 7%  $\epsilon$ -proteobacteria, 5% unclassified *proteobacteria*, 2%  $\delta$ -proteobacteria, and 2%  $\beta$ -proteobacteria. The remaining bacterial diversity was spread across the *Deinococcus–Thermus* (8%), *Aquificae* (6%) and *Bacteroidetes* (5%) phyla, and 4% shared in other phyla. The Archaeal diversity (0.4%) was composed of *Thermoproteales*, *Thermococcales*, and *Archaeoglobales*.

The evolution of archaeal diversity on the successive enrichment cultures over the 3 weeks was dominated by the *Euryarchaeota* phyla (mainly the orders *Archaeoglobaceae* and *Thermococcaceae*), which rose from 0.4% in the initial sample to > 70% in week 3, whether on the electrode or in liquid media. Interestingly, at genus level, dominant archaeal OTUs on the electrode were closely related to uncultured *Thermococcus* species (22% (week 1), 32% (week 2), and 56% (week 3)) and *Geoglobus* spp. (0.2% (week 1), 22% (week 2), and 18% (week 3)) affiliated to *Geoglobus ahangari* (99% identity on ~ 300 bp 16S rDNA fragments). Moreover, planktonic microbial diversity was mainly enriched in uncultured *Thermococcus* species (4.9% (week 1), 46.5% (week 2), and 72.2% (week 3)) rather than *Geoglobus* spp. (< 0.7% during the 3 weeks).

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Bacterial diversity over the first 3 weeks on the electrode and in liquid media was mainly composed of species belonging to the order *Bacillales* (representing up to 94% of biodiversity in liquid media during week 1). The remaining OTUs, mainly found on electrodes, were surprisingly distributed among the mainly mesophilic orders *Burkholderiales*, *Pseudomonadales*, *Xanthomonadales*, and *Sphingomonadales*, which were not expected in these hyperthermophilic conditions. However, most of these phylogenetic orders harbor some thermophilic or hyperthermophilic species [33, 34] and could potentially be exoelectrogens. Note the gradual decrease in proportion of these bacteria on the electrode, which fell to less than 15% of cumulative total by week 3, indicating lower competitiveness in our conditions compared with *Thermococcus*, *Geoglobus*, and *Bacillales* species.

In order to identify members of the community requiring the electrode as electron acceptor for their growth, a comparative enrichment without electron acceptor was performed in parallel with week 2 enrichment. We carried out an open-circuit control with week 1 final culture medium as inoculum and with the electrode as inert support without the possibility of electron exchange with the counter electrode. This control enrichment showed the following major phyla (on the electrode and liquid media, respectively): *Proteobacteria* (21% and 0.18%), *Firmicutes* (52% and 77%), and *Euryarchaeota* (15% and 22%). Interestingly, based on average abundance analysis, microbial biodiversity was richer on the electrode than in the liquid media (see the richness and evenness indexes in Fig. 3). The major members of the *Proteobacteria* phylum, *Burkholderiales* (10%), *Enterobacteriales* (7%), and *Sphingomonadales* (3%) orders, thus prefer to grow into biofilm than planktonic

cells. At order level, *Bacillales* (40%), *Clostridiales* (9%), and *Lactobacillales* (3%) were the remaining major bacterial orders growing on the electrode. However, only the archaeal order *Thermococcales* (15%) was present in this enrichment control, whether on the electrode or in liquid media. No OTUs of the order *Archeoglobales* were observed. Microbial diversity in the liquid media was mainly composed of *Bacillales* (73%), and *Thermococcales* (22%), which are known to be fermentative microorganisms.

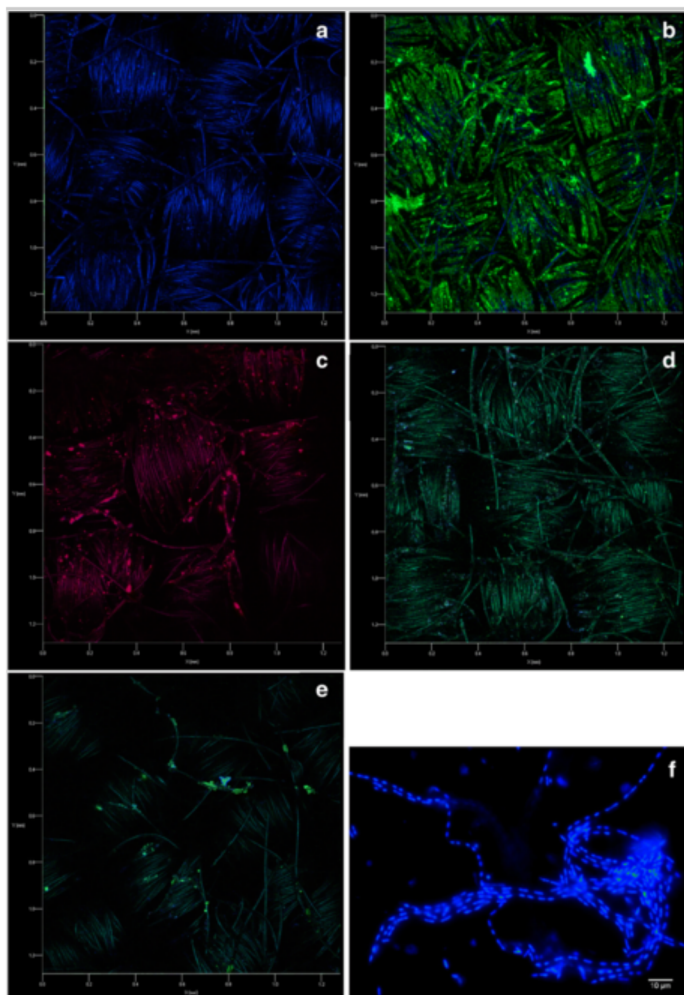
## Microscopic Observation of Exoelectrogenic Biofilm

The HEB obtained during the second week was observed under a confocal microscope with DNA staining using (i) Syto9 as fluorescent nucleic acid stain and (ii) FISH probes specific to *Bacteria*, *Archaea*, *Thermococcales*, and *Archeoglobales* on separate observations. Figure 4 presents the micrographs obtained on the Syto9 abiotic control (Fig. 4a) with only reflectance from the carbon fibers (blue color) and the week 2 anode (Fig. 4b) with total cell staining (green color) covering the anode's carbon fibers. A hyperthermophilic biofilm was observable on the electrode surface, with extracellular polymeric substances (EPS) most likely bridging the spaces between carbon fibers. The *Archaea*-specific FISH probe (Fig. 4c) revealed two morphologies of biofilm distribution, with a diffuse signal all over the carbon fibers and concentrated clusters distributed on the fiber surfaces. FISH probes specific to the main archaeal taxonomic orders found in 16S barcoding analyses attributed the diffuse presence all over the carbon fibers to *Archeoglobales* (green color, Fig. 4d) and concentrated clusters to *Thermococcales* (green color, Fig. 4e). The *Bacteria*-specific FISH probe did not produce enough signal to visibly identify bacterial distribution on the electrode. However, some cells were visible on carbon fibers when the  $\times 63$  (NA1, 4PLAN APO) objective was used. Note that the important *Bacillales* proportion found on the electrode (Fig. 3) during week 2 was skewed by the higher 16S gene copy number present in species belonging to *Bacillaceae* (10 copies on average; rrnDB, Stoddard et al., 2014) compared with *Archaea* (1 copy on average). On the other hand, microscopic observation of DAPI-stained filtrated liquid media (Fig. 4f) showed the presence of long-chained bacilli cells and some irregularly shaped coccoid species, corresponding most probably to *Bacillus* sp. and *Thermococcus* sp., respectively.

### Fig. 4

Confocal microscopic observation with Syto9 of abiotic control electrode (a) after 1 week of culture (a) and biofilm (b) on the end of the second week of culture (b). FISH observation with *Archaea* (red) (c), *Archeoglobales* (green) (d), and *Thermococcales* (green) (e) probes. Three-dimensional top projection of  $1.2 \times 1.2$  mm patch of anode at  $\times 100$  magnification. Epifluorescent observation of liquid media of the second week of culture (f) at  $\times 1000$  magnification with DAPI

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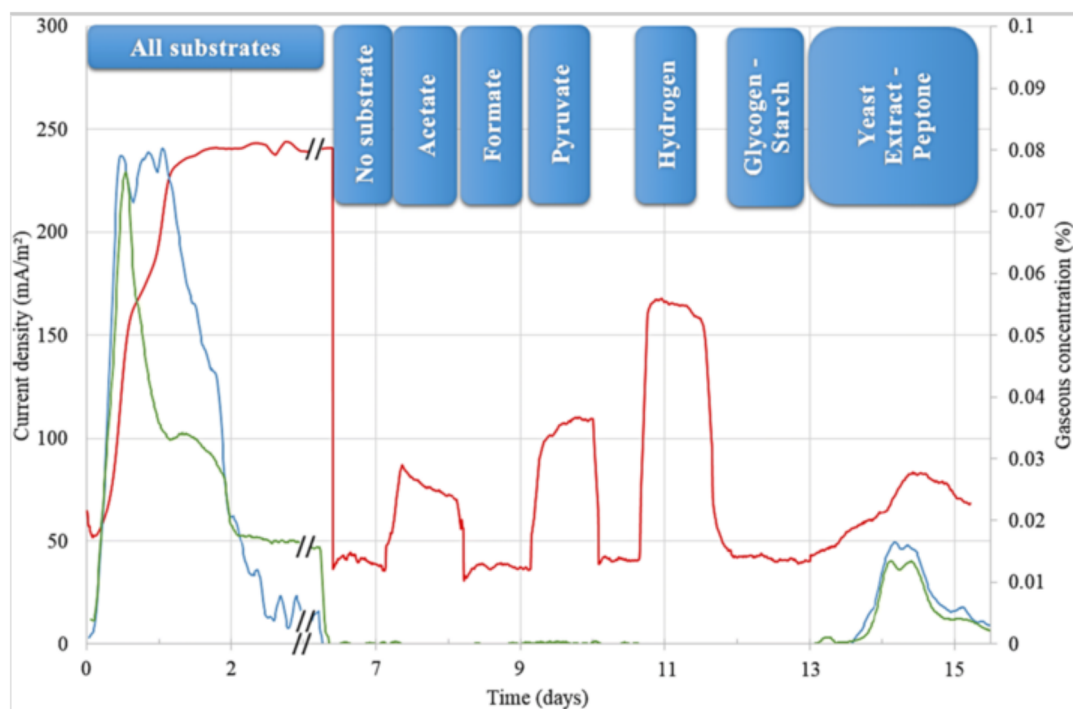
AQ6

### Current Density Production with a Single Substrate

During the fourth week, current production was measured on each separate substrate in fresh media in order to identify the metabolic interactions and substrate specificities involved in the exoelectrogenic process. Figure 5 reports current density and gas production for each substrate, highlighting the immediate current density production with 10-mM acetate ( $80 \text{ mA m}^{-2}$ ), 10 mM of pyruvate ( $108 \text{ mA m}^{-2}$ ), and a flow rate of  $50 \text{ mL min}^{-1}$  of  $\text{H}_2$  ( $165 \text{ mA m}^{-2}$ ) compared with abiotic controls in the same conditions. The current density on  $\text{H}_2$  is certainly higher than in normal fermentative condition, due to high rate of  $\text{H}_2$  sparged in the bioreactor to overcome potential diffusion limitation. Interestingly, there was a gradual increase of current with a mix of  $0.5\text{-g L}^{-1}$  yeast extract and  $0.5\text{-g L}^{-1}$  peptone (up to  $83 \text{ mA m}^{-2}$ ), correlated with the weak production of  $\text{CO}_2$ ,  $\text{H}_2$ , and acetate. This gradual increase suggests an indirect use of fermentation products by the on-electrode HEB. No current was obtained on formate and polysaccharides.

#### Fig. 5

Current density (red line), hydrogen (green line) and carbon dioxide (blue line) production over time by hyperthermophilic exoelectrogenic biofilm on the anode of a MFC at  $80 \text{ }^\circ\text{C}$  under rich (from 0 to 7 days) and specific substrate conditions (from 7 to 15 days)



## Discussion

### Hyperthermophilic Exoelectrogenic Enrichments in MFC

Despite the discovery, more than 8 years ago, of the electrically conductive property of the chimney wall of hydrothermal vents [12], there has been relatively little investigation yet of microorganisms able to use this property for their metabolism, nor of their role in the trophic chain of the biosphere. We previously demonstrated the presence of hyperthermophilic exoelectrogenic communities from a hydrothermal vent using an enrichment method with acetate as sole energy source and electrode as electron acceptor in an MEC (Pillot et al., 2018). To gain further insight into hyperthermophilic exoelectrogenic diversity and its trophic interactions in the deep-sea hydrothermal ecosystem, a MFC was inoculated with chimney sample with a rich diversity of electron donors. Given the current production in the MFC inoculated with chimney sample (Fig. 1), the increase of capacitive and catalytic currents on cyclic voltammograms (Fig. 2), the microscopic observation of a biofilm on the electrode surface (Fig. 4), and the increase of bacterial and archaeal 16S rRNA gene copies on the electrode (see Supplementary Fig. 4), it could be concluded that a HEB was enriched in our system. The quick electrode change each week allowed to select the exoelectrogenic biodiversity colonizing the electrode and other tightly related microorganisms (Fig. 3), increasing the current production and decreasing the lag time before current production. This selection phenomenon is commonly observed with the enrichment of exoelectrogenic communities from environmental samples [35, 36].

### Microbial Composition of the Biofilm Community

*Thermococcales*, *Archaeoglobales*, and *Bacillales* are the three dominant orders retrieved on the electrode surface and in liquid media over each week of enrichment (Fig. 3). Interestingly, all three orders had already been retrieved as the main representatives of exoelectrogenic biofilm diversity in a previous study in more selective conditions, inoculated with sample from another hydrothermal field [10]. Thus, the fact that little more biodiversity was enriched in far more different substrate and electrode potential conditions seems to indicate that species of these three orders are potentially (i) the sole hyperthermophilic exoelectrogenic species in those environments, (ii) the sole in obligatory close relation with exoelectrogenic species, or (iii) the most competitive in such conditions.

*Thermococcales* are hyperthermophilic archaea known to mainly grow by fermentation of peptides or carbohydrates. The final fermentation products are mainly acetate, hydrogen, and CO<sub>2</sub>. They are recognized as ubiquitous to hydrothermal vents and easily come to dominate these environments in fermentative conditions [37]. Here, despite their colonization as clusters on the carbon fibers of the electrode (Fig. 4e), we were unable to determine the exact role of *Thermococcales* in electric current production. However, some *Thermococcales*

species, like strains of *Pyrococcus furiosus*, have been shown to produce electric current in MFCs [9] or to reduce insoluble iron oxide through EET [38, 39].

The *Archeoglobales* present all over the carbon fibers of the anode (Fig. 4d) are mainly affiliated to *Geoglobus* spp. (98.6% similarity with *Geoglobus ahangari*). *Geoglobus* species are hyperthermophilic archaea known to grow lithoautotrophically on H<sub>2</sub> and CO<sub>2</sub> or heterotrophically on volatile fatty acids and peptides as electron donors and carbon source, and to obligatorily use soluble or insoluble Fe (III) as electron acceptor [40, 41]. Recent studies have shown that *Geoglobus ahangari* has exoelectrogenic capacity on anodes when oxidizing acetate, whereas it did not produce current with H<sub>2</sub> [11]. Moreover, a *Geoglobus* species was found in the hyperthermophilic community on an MEC inoculated with hydrothermal chimney sample [10]. This is in good agreement with specific enrichment of *Geoglobus* spp. on the electrode over time and the absence of *Geoglobus* spp. enrichment in the open-circuit control. Indeed, these results were fully coherent with the fact that *Geoglobus* spp. needs the electrode as electron acceptor.

The *Bacillales* enriched in our experiment, mainly in liquid media (Fig. 3, Supplementary Fig. 2, Fig. 4), were affiliated to *Bacillus* and *Geobacillus* species. *Bacillus* are Gram-positive sporulating rod-shaped bacteria frequently found in marine sediments or shallow hydrothermal vents. They are known to degrade a wide range of substrates, including peptide and polysaccharides. All *Bacillus* spp. grow aerobically but some have the ability to undergo fermentation or anaerobic respiration on nitrate. Most *Bacillus* spp. are mesophilic, with optimum temperature between 15 to 55 °C, but former thermophilic *Bacillus* were reclassified as *Geobacillus* in 2001 and can grow from 55 to 80 °C [42]. Moreover, microscopic observation of high amounts of bacilli chains in liquid media (Fig. 4f), typical of *Bacillus* spp., tends to confirm the enrichment of hyperthermophilic members of *Bacillales*. We suppose this strain can either perform fermentation or potentially use the electrode as alternative electron acceptor. Indeed, different species of *Bacillus* and *Geobacillus* have already been shown to produce electricity in MFCs, on polysaccharides [43], on glucose [44, 45], or on acetate [30]. However, whatever the species, an electron shuttle had to be added or endogenously produced by the bacteria to generate some amount of current. In our study, as no current was observed on the control experiment on filtered spent media with sterile electrode, we can reject the hypothesis of mediated electron transfer in our experiments.

The biodiversity obtained in the open-circuit control inoculated with week 1 spent medium demonstrated that the connected electrode plays a specific role in microbial composition. Indeed, *Geoglobus* sp. was totally extinguished on the electrode and liquid media yet was enriched on the connected electrode in week 2. This could be directly correlated with the fact that they require an electron acceptor for growth. *Thermococcales* and *Bacillales*, both fermentative microbes, were still equally represented on the electrode as in the liquid media of the open-circuit control. Finally, the conservation of more biodiversity (higher evenness index, Fig. 3) on solid support than in liquid media can be related to the protective role of biofilm colonizing conductive or inert supports [46].

Otherwise, the results show similar amount of current density between our experiments and in the literature while the on-electrode biodiversity was different. This suggests that the maximum of current production is not necessarily community-dependant but more related to the total surface area colonized by exoelectrogens.

## Trophic Relation Between Fermentative and Exoelectrogenic Microorganisms

In order to identify the role of each electron donor in current production and in the trophic chain of the community, each one was tested separately on the mature biofilm of week 4 enrichment. These tests showed no current production on polysaccharides while most *Bacillales*, and a few *Thermococcus* sp., have the ability to hydrolyze starch and ferment glucose. This could be explained by the renewal of the liquid media, removing planktonic fermentatives such as *Bacillales* and *Thermococcus*, or by their lack of the enzymatic machinery to hydrolyze polysaccharides, as reported in some *Thermococcus* sp. [47, 48].

However, we confirm the presence of direct respiratory metabolism on the electrode from acetate, pyruvate, and H<sub>2</sub>. *Geoglobus* spp., present on the electrode, would be the only known species able to produce current from these substrates. Indeed, *Geoglobus* spp. are known to metabolize these substrates and grow using iron oxide particles as electron acceptor [41]. However, a previous study found that *Geoglobus ahangari* was unable to produce current on H<sub>2</sub> [11]. Sequencing of the ~300-bp hypervariable region V4-V5 of the gene coding for 16S rRNA failed to precisely affiliate the OTUs obtained with the species *Geoglobus ahangari*. Thus, we are

potentially in the presence of several new exoelectrogenic species belonging to the *Geoglobus* genus. Furthermore, the presence of a microbial community in our experiment can allow microbial interactions, and thereby increase the range of metabolic capacity. However, this is the first time that direct biotic current production has been observed on H<sub>2</sub> in a hyperthermophilic MFC. Immediate current production in presence of a fermentation product such as pyruvate, acetate, and hydrogen suggests a community already adapted to the direct oxidation of this substrate on the electrode. In contrast, we observed a gradual and slow increase of current production with a complex substrate such as peptone and yeast extract. This suggests that a first fermentation step is necessary in order to release small molecules readily oxidizable on the anode. This hypothesis is supported by the observation of higher production of CO<sub>2</sub> and hydrogen released in the open-circuit control when no final electrode acceptor was available (see Supplementary Fig. 4). These trophic relations were also observed in co-cultures of different species of *Thermococcus*—producing H<sub>2</sub> and CO<sub>2</sub> from fermentable compounds—with *G. ahangari*—consuming H<sub>2</sub> and CO<sub>2</sub> to produce fermentable compounds (unpublished data). Note that indirect current production in a thermophilic MFC was already observed from the autotrophic community conversion of syngas (composed of H<sub>2</sub> and CO<sub>2</sub>) to acetate which is then used as electron donor by exoelectrogenic biofilm to produce current [49].

Taken together, these results support the hypothesis of trophic interactions between fermentative and exoelectrogenic microbes. In the environment, this syntrophic relationship would allow carbon cycling inside hydrothermal vent biofilms. Indeed, the surrounding organic compound released by living and dead organisms (substituted by peptone and yeast extract in our experiment) would be metabolized by fermentative microorganisms like *Thermococcus* spp. and *Bacillus* spp. (present as clusters in our biofilm in Fig. 4e or freely dispersed in the seawater) producing hydrogen, carbon dioxide, and organic acids. Indeed, some *Thermococcus* species can use microbial-origin extrapolymeric substances (dextran, pullulan, peptides, etc.) for fermentative growth [50]. These fermentation products may then be used as electron donor (acetate, H<sub>2</sub>) and carbon source (CO<sub>2</sub>) for exoelectrogenic microorganisms, such as *Geoglobus* species (covering the carbon fibers in Fig. 4d), which would breath on the conductive pyrite composing the chimney wall and contribute to the abiotic electric current previously observed [20]. Finally, these (autotrophic) exoelectrogens may potentially release organic compounds like EPS in the biofilm that can be later reused by fermentative microbes. This potential cross-feeding phenomenon could allow the survival, in oligotrophic and extremely unstable conditions, of these microbes grouped as biofilm on the hydrothermal chimney surface by concentrating and recycling the organic matter inside the biofilm.

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## Compliance with Ethical Standards

*Conflict of Interest* The authors declare that they have no conflict of interest.

## Electronic supplementary material

**ESM 1**      **ESM 2**      **ESM 3**      **ESM 4**      **ESM 5**  
(DOCX 16 kb) (DOCX 16 kb) (DOCX 66 kb) (DOCX 15 kb) (PDF 446 kb)

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