

Interspecies interactions mediated by conductive minerals in the sediments of the Iron rich Meromictic Lake La Cruz, Spain

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† Deceased. This article is *in memoriam* of Professor Maria R. Miracle.

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ABSTRACT

Interspecies interactions mediated by conductive minerals in the sediments of the Iron rich Meromictic Lake La Cruz, Spain

Lake La Cruz is considered a biogeochemical analogue to early Earth marine environments because its water column is depleted in sulfate, but rich in methane and iron, similar to conditions envisaged for much of the Precambrian. Here we show that conductive particles drove the metabolic coupling between electroactive microbial clades from this environment. The anoxic sediment of Lake La Cruz was rich in biogeochemically 'reactive' iron minerals, and harbored known electroactive species such as *Geobacter* and *Methanotrix*, in addition to groups never linked to an electroactive lifestyle. Slurry incubations on various substrates in the presence of conductive particles showed 2 to 4 times higher methanogenic activity, as compared to incubations with non-conductive glass beads or without added particles. In the absence of conductive particles, all tested substrates were metabolized to acetate, which accumulated above 8 mM depending on substrate (8 ± 0.6 to 11.7 ± 1.2 mM). Only by enabling syntrophic acetate oxidation with conductive minerals could we prevent acetate accumulation. Acetate oxidation conductively coupled to methanogenic activity had a stoichiometric recovery of 70 % and could be maintained in subsequent transfers only if amended with conductive particles. Mud-free enrichments without conductive particles ceased any metabolic activity after the second transfer. Conductive particles preserved a consortium of *Youngiibacter*-*Methanotrix*, whereas without conductive particles *Youngiibacter* spp. died off. Syntrophic consortia from this early Earth analogue environment only survived in the presence of conductive particles inferring that minerals may have arbitrated the earliest interspecies associations.

Key words: *Methanotrix*, *Geobacter*, *Youngiibacter*, magnetite, conductive particles, granular activated carbon, direct interspecies electron transfer, mineral mediated syntrophy, iron meromictic lake

RESUMEN

Interacciones interespecíficas mediadas por minerales conductivos en el sedimento del Lago La Cruz (España), un lago meromictico rico en hierro

La laguna de La Cruz puede considerarse como un análogo biogeoquímico de los ambientes marinos en la Tierra primitiva, porque su columna de agua, muy escasa en sulfato pero rica en metano y hierro, presenta condiciones similares a estos ambientes en el Precámbrico. En este ambiente encontramos que partículas conductivas establecen un acoplamiento metabólico

entre clados microbianos electroactivos. Demostraremos que el sedimento anóxico de la laguna de La Cruz, rico en minerales de hierro biogeoquímicamente “reactivos”, alberga conocidas especies electroactivas tales como *Geobacter* y *Methanothrix*, además de otros grupos microbianos que no han sido previamente asociados con un estilo de vida electroactivo. Las incubaciones de lodo en varios sustratos realizadas en presencia de partículas conductivas tuvieron como resultado una actividad metanogénica de dos a cuatro veces mayor, comparadas con las incubaciones con microperlas de vidrio no conductivas o sin partículas adicionadas. En ausencia de partículas conductivas, todos los sustratos probados fueron metabolizados a acetato, que se acumulaba hasta una concentración de hasta aproximadamente 8 mM dependiendo del sustrato (8 ± 0.6 to 11.7 ± 1.2 mM). Sólo la adición de mineral conductivo a los lodos de la laguna de La Cruz hacía posible la oxidación del acetato, impidiendo su acumulación. La oxidación del acetato acoplada a una alta actividad metanogénica tuvo una recuperación estequiométrica del 70 % y pudo mantenerse en sucesivos enriquecimientos libres de sedimento solo si estos se adicionaban con minerales conductivos. Por el contrario, enriquecimientos libres de sedimento y sin partículas conductivas cesaron cualquier actividad metabólica en la segunda transferencia de la serie. Las partículas conductivas conservaron la existencia del consorcio *Youngiibacter*-*Methanothrix*, mientras que *Youngiibacter* spp. muere y desaparece en ausencia de las partículas conductivas. Consorcios sintroficos de este ambiente análogo al de la Tierra primitiva sólo sobrevivieron en presencia de partículas conductivas infiriendo que los minerales pueden haber mediado en las asociaciones interespecíficas más primitivas.

Palabras clave: *Methanothrix*, *Geobacter*, *Youngiibacter*, magnetita, partículas conductivas, carbón activo granulado, transferencia directa de electrones interespecífica, sintrofia con mediación mineral, lago meromítico ferruginoso

INTRODUCTION

It has been proposed that eukaryotic life arose from syntrophic interactions between Deltaproteobacteria and methanogenic archaea (Moreira & Lopez-García, 1998; López-García & Moreira, 1999; Martin & Russell, 2003) in the anoxic and ferruginous (Fe-rich) early Archaean ocean (Crowe *et al.*, 2008). Similar conditions exist today in the anoxic deeper waters of some lakes (Crowe *et al.* 2008; Bura-Nakic *et al.*, 2009; Llíros *et al.*, 2015), including Lake La Cruz, Spain (Walter *et al.*, 2014; Camacho *et al.*, 2017). Most studies of these environments have focused on the phototrophic and methanotrophic communities in the water column, yet little attention has been given to either the methanogenic community buried in the sediments or the possible impact of iron-minerals on their physiology. Only recently, have researchers investigated the methanogenic community from Lake Matano, Indonesia which displayed high methanogenic rates when spiked with the iron-oxide, goethite (Bray *et al.*, 2017). However, the possibility of a mineral-mediated syntrophic interaction was not assessed.

Generally, syntrophic associations are carried out indirectly, in which case electron transfer between partners is assisted by diffusible chemicals (H_2 , formate, shuttles). These classical syntrophic interactions require a bacterium and a methanogenic archaeon as syntrophic partner.

The bacterium oxidizes complex organics to reduced compounds (i.e. H_2), that are then retrieved by the methanogenic archaeon in order to reduce CO_2 to methane (Shrestha & Rotaru, 2014). Recent studies have shown that, sometimes, interspecies electron transfer does not require a diffusible chemical carrier. In the absence of a diffusible electron carrier, interspecies electron transfer could occur via conductive particles (magnetite, chars, pyrite) (Liu *et al.*, 2012; Chen *et al.*, 2014; Liu *et al.*, 2015; Kato & Igarashi, 2018; Wang *et al.*, 2018) or directly by forging electric connections via a self-assembled extracellular network of conductive pili and *c*-type cytochromes between the two syntrophic partners (Summers *et al.*, 2010; Rotaru *et al.*, 2014b, 2014a). The later is known as direct interspecies electron transfer (DIET). DIET was shown to be accelerated by conductive materials possibly because cells save energy by pausing the production of their own conductive extracellular network (Liu *et al.*, 2015; Wang *et al.*, 2018). Consequently, mineral-mediated syntrophy is energetically more favorable than the usual syntrophic associations.

It has been proposed that Fe-minerals such as pyrite helped nucleate the membranes of the earliest cells (Wächtershäuser, 1988a; Russell *et al.*, 1994). Many membrane bound proteins involved in electron transfer through the membranes of present day cells contain FeS centers

(i.e. ferredoxins). It is therefore likely that some of the earliest FeS proteins might have played a role in electron transfer between cells.

It has been speculated that conductive-minerals also mediate the interaction between protocells with leaky cell walls present in the mineral-rich Archaean ocean (Lane & Martin, 2012). Interactions between cells with different metabolisms are thought to be at the origin of eukaryogenesis, as such cells compartmentalized the functions within the eukaryotic cell (Moreira & Lopez-Garcia, 1998; López-García & Moreira, 1999; Martin & Russell, 2003). In the present study, we investigated the conductive iron-mineral dependency of interspecies interactions between bacteria and methanogens from the sediments of the Fe-rich, stratified Lake La Cruz. Specifically, we were interested in whether reactive Fe minerals would support conductive-mineral mediated interspecies

interactions. As the biogeochemical setting of the lake makes it a prime early analogue (Walter *et al.*, 2014, Camacho *et al.*, 2017), we also discuss mineral-mediated syntrophy as a relic of ancestral associations.

MATERIAL AND METHODS

Sampling and incubations

During an expedition at Lake La Cruz in central Spain (Fig. 1) in September 2014, we sampled the lake water and sediment. Lake La Cruz is a permanently stratified, meromictic, doline lake located in a karst region in the Iberian Mountain Range. The lake is circular with a diameter of 122 m. At the time of sampling, the maximum depth was 20 m and the chemocline started at ~12 m depth. Water samples were pumped from depths above,

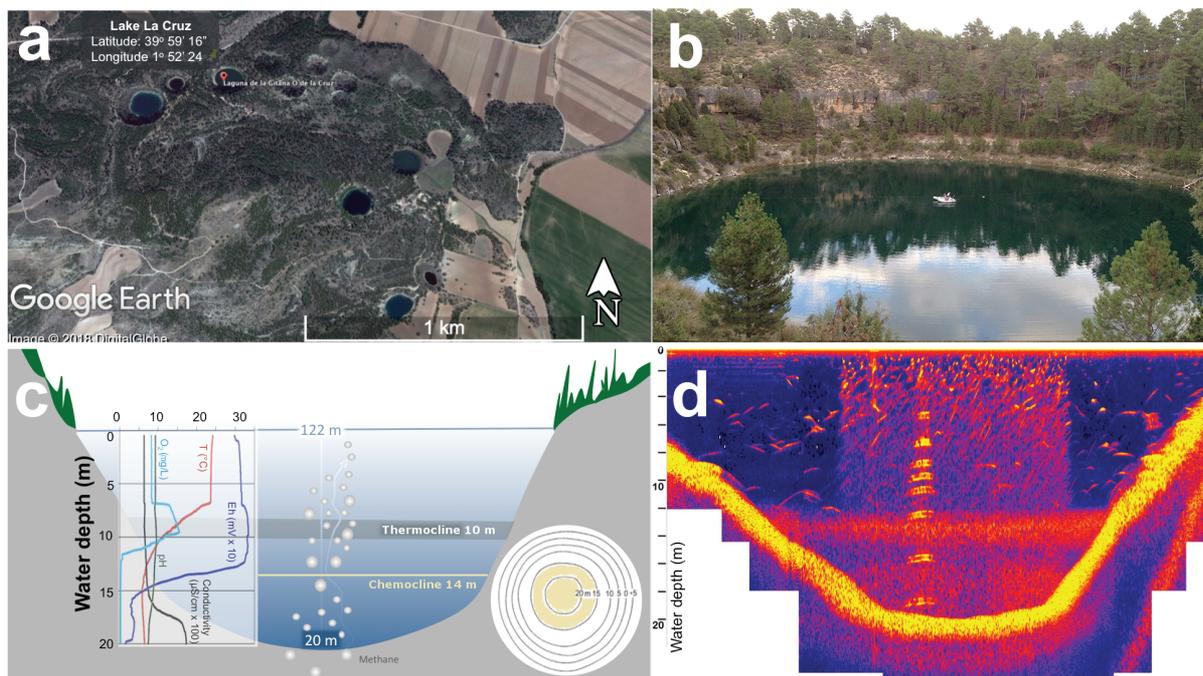


Figure 1. Lake La Cruz with its geophysical features. Map of the Cuenca lake area with geographical coordinates (a); and an image of the sampling site - lake La Cruz (b). Schematic representation of the lake (c) including a bathymetric map (c-round inset), and *in situ* physico-chemical characteristics of the water column (c-graphic inset: — conductivity $\mu\text{S}/\text{cm}$, — temperature $^{\circ}\text{C}$, — oxygen mg/L , — pH, — Eh mV). An echogram indicating the chemocline from 12-14 m, with visible gas ebullition in the central area (d). *La laguna de La Cruz con sus características geofísicas. Mapa de la zona de Cuenca con coordenadas geográficas del lago estudiado (a); y una imagen del lugar de muestreo – laguna de La Cruz (b). Representación esquemática del lago (c) que incluye un mapa batimétrico y las características físico-químicas in situ en la columna de agua: (— conductividad $\mu\text{S}/\text{cm}$, — temperatura $^{\circ}\text{C}$, — oxígeno mg/L , — pH, — Eh mV). El ecograma muestra la quimiocline situada a 12-14 m, con ebullición gaseosa visible en la zona central (d).*

within, and below the chemocline at the deepest part of the lake from a boat tethered from shore to shore of the lake. The pumping apparatus was designed to withdraw water samples without contact with the atmosphere, and both the apparatus and sampling protocol have previously been described in detail (Miracle *et al.*, 1992; Posth *et al.*, 2017). Samples were gathered and fixed directly on the boat and stored until analysis in the lab.

Three sediment cores were collected from the center and the deepest part of the lake (coordinates: 1° 52' 24" West; 39° 59' 16" North, Fig 1) using a sediment corer (Kajak sediment core, KC Denmark). The cores (50 cm length × 7 cm diameter) were sealed without air bubbles as they were pulled up from depth with rubber stoppers immediately inserted to avoid exposure to the atmosphere. Within 24 hours of sampling, the sediment was partitioned into depth intervals, and fixed for biogeochemical and molecular analyses inside an N₂-filled inflatable glove bag, as described in detail below.

For downstream incubations, sediment from 0-15 cm depth was sampled and placed in Duran bottles secured with butyl-rubber stoppers, with a headspace of 2 bars N₂:CO₂ 80:20 mix. Samples were stored at 4 °C until later used for incubations.

Slurries were prepared in an N₂-filled anaerobic chamber in the laboratory. For these slurries, we used 3 mL cut-off syringes to distribute 2.5 mL of sediment into 20 mL gas-tight vials filled with 7.5 mL of medium, either modified DSM 120 or DSM 334. Modified DSM 120 medium was prepared as described previously (Rotaru *et al.*, 2014b), but with 0.6 g/L NaCl. Three successive ten-fold dilutions of the sediment slurries led to essentially mud-free enrichments in which sediment particles could not be detected visually or by microscopy. Before inoculation, the complete medium, which lacked the substrate and (semi)conductive minerals, was dispensed anaerobically by syringe into sterile degassed vials with or without minerals prepared as below.

Two electrically conductive particle types (granular activated carbon and magnetite) were selected to be tested because they were previously confirmed to stimulate DIET in methanogenic co-cultures (Liu *et al.*, 2012; Zheng *et al.*, 2017).

Granular activated carbon (GAC, Sigma Aldrich) had a particle size between 180 and 300 µm diameter and estimated conductivity of circa 1000 S/m (Kastening *et al.*, 1997), and magnetite (Sigma Aldrich) with particles less than 5 µm diameter, and estimated electrical conductivity ranging between 0.1 and 1 S/m (Rochelle & Schwertmann, 2003; Blaney, 2012). Both materials have conductivities similar or higher than the pili that carry out extracellular electron transfer in *Geobacter sulfurreducens* (5 S/m (Adhikari *et al.*, 2016)). We weighed 0.1 g/L of each material, added to vials, overlaid with 200 µl ultrapure water for wet sterilization, degassed for 3 minutes with N₂:CO₂ 80:20 mix, and autoclaved at 121 °C for 25 min. Controls with non-conductive particles were setup with acid-washed glass beads (less than 105 µm diameter) instead of conductive minerals. Substrates (5 mM glucose, 5 mM sodium butyrate, 10 mM sodium acetate, 10 mM ethanol) were added to media from sterile anoxic 1 M stocks using aseptic and anaerobic techniques. Controls without electron donors were carried out in order to identify whether the organics in the sediment served as substrates for methanogenesis. All incubations were carried out at room temperature (20-23 °C) in triplicate, unless otherwise stated.

Gas samples were withdrawn, stored anaerobically and then analyzed for methane on a Thermo Scientific gas chromatograph (Rotaru *et al.*, 2018). To test for short chain volatile fatty acids (SCVFA) we used high performance liquid chromatography (HPLC) as described elsewhere (Rotaru *et al.*, 2018).

Biogeochemistry

For biogeochemical parameters, we took water column samples at different depths and sampled the sediment obtained via the gravity corer. Geochemical parameters of relevance to this work were methane, soluble ferrous iron, and particulate reactive iron mineral species. We will use the term reactive iron species to refer to oxalate, dithionite and HCl soluble iron oxides and sulfides (Poulton *et al.*, 2004).

Water column methane was sampled from the pumping apparatus through chemically resistant

tubing (VWRs ISO-VERSNIC®) into 20 mL glass, GC vials (Supelco, Sigma-Aldrich). For each sampling depth, 5 mL samples in triplicate were added to GC vials pre-doped with 10 mL 2 N NaOH to retain CO₂ in the liquid phase. The vials were sealed with butyl-viton rubber stoppers, and stored upside down in the dark at 4 °C until analysis.

Sediment methane concentrations were determined from sediment slices extracted every 2 cm in an anoxic glove bag filled with N₂ gas.

Our measurements of available electron acceptors at the sediment boundary layer corroborated previous investigations during summer months at this lake (Miracle *et al.*, 1992; Walter *et al.*, 2014; Camacho *et al.*, 2017) and showed a depletion of sulfate and Fe³⁺ (< 10 µM sulfate, < 1 µM Fe³⁺) and no detection of oxygen and nitrate. Thus sediments mainly relied on methanogenesis for decomposition of organic matter below the water-sediment boundary. For sedimentary methane determination, sliced sediment was filled into glass GC vials, to which 1 M (2.5 %) NaOH was added in order to stop any additional microbial activity. The vials were capped with butyl-viton stoppers, crimped, and inverted until lab analysis. Sedimentary methane concentrations were determined on a Perkin Elmer GC, as previously described (Rotaru *et al.*, 2018).

Porewater was analysed for reduced iron concentrations at ~2 cm depth resolution after extraction using Rhizons (Rhizosphere; pore size 0.2 µm) inside a glove bag with an N₂-atmosphere. Dissolved Fe²⁺ was determined immediately using the ferrozine assay (Lovley & Phillips, 1987; Viollier *et al.*, 2000).

To determine iron mineral speciation, sediment was subsampled at each 2 cm-depth interval and stored at -20 °C. Reactive iron species (dithionite and HCl soluble iron species) were identified from freeze-dried samples stored at -20 °C by applying a modified sequential iron extraction procedure (Poulton & Canfield, 2005). In the first step, a room temperature 0.5 N HCl extraction was applied to dissolve poorly crystalline iron oxides such as ferrihydrite, surface absorbed Fe²⁺, iron carbonate minerals such as siderite, and acid volatile iron monosulfides (Zegeye *et al.*, 2012). Subsequently, a pH 4.8

sodium dithionite extraction was employed to dissolve crystalline ferric oxide minerals such as goethite and hematite, followed by an oxalate extraction to dissolve magnetite (Poulton & Canfield, 2005). The total concentration of iron dissolved in each operationally defined extraction phase was determined by flame atomic absorption spectroscopy (AAS). For the 0.5 N HCl extraction, dissolved Fe²⁺ was also measured immediately via the ferrozine assay (Lovley & Phillips, 1987). Extraction of this Fe²⁺ from the total Fe determined for this extraction by AAS gave the Fe³⁺ concentration associated with poorly crystalline iron oxides such as ferrihydrite. Iron sulfide phases were determined via a two-step sequential extraction procedure (Canfield *et al.*, 1986). Acid volatile sulfide minerals (FeS) were determined by extraction with hot 6 N HCl under N₂, with the released sulfide trapped as Ag₂S. Pyrite (FeS₂) was then determined after addition of chromous chloride, with the sulfide again trapped separately as Ag₂S. After filtration, the concentrations of Fe in FeS and FeS₂ were determined stoichiometrically. The concentration of Fe present as FeS was subtracted from the Fe²⁺ concentration determined by the 0.5 N HCl extractions, to give surface reduced and carbonate-associated Fe²⁺. Replicate extractions gave a RSD of < 5 % for all phases.

Scanning electron microscopy

Samples from the water column were preserved in 4 % formalin, filtered on Nucleopore carbonate filters, with a pore size of 0.2 µm, and dehydrated in 20 min. steps with ethyl alcohol (30 %, 50 %, 70 %, 90 % and two times 100 %). Then the samples were critical point dried prior to palladium/gold sputter coating and visualization on a Hitachi S-4800 FE scanning electron microscope ran at an electron beam acceleration voltage of 20 kV.

Molecular analyses

For molecular analyses we sampled 2 mL of sediment at 2 cm depth resolution using cut-off syringes inside a N₂-filled glove bag. Sediment was pooled together every 4 cm and fixed with MoBio RNAlater 1:1 v/v (Rotaru *et al.*, 2018).

Prior to DNA extractions, most of the RNAlater was removed by centrifugation. For DNA extraction we used the top 16 cm of sediment from triplicate cores. Extractions were carried independently for each core with the MoBio RNA Soil kit coupled to the MoBio complementary DNA Soil kit, following the manufacturer's protocol. DNA was quantified using a Nano Drop prior to downstream applications. The DNA extracted from each core was amplified with the following primer pair S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18, which according to Klindworth *et al.* (2013), was the best for MiSeq

amplicon sequencing, targeting more than 89 % of Bacteria and more than 88 % of Archaea. PCR amplification and indexing (using Nextera XT index kit, Illumina) of the PCR products for the triplicate samples was conducted following the Illumina 16S rRNA gene amplicon sequencing protocol (Illumina, USA). The DNA samples were then sequenced using $\times 300$ PE MiSeq sequencing approach at Macrogen (www.macrogen.com), using Illumina's protocol. The sequences generated circa 1 million reads for each core, which were imported into CD-HIT-OTU to remove noisy data and clustered into OTUs, using a 97 % species

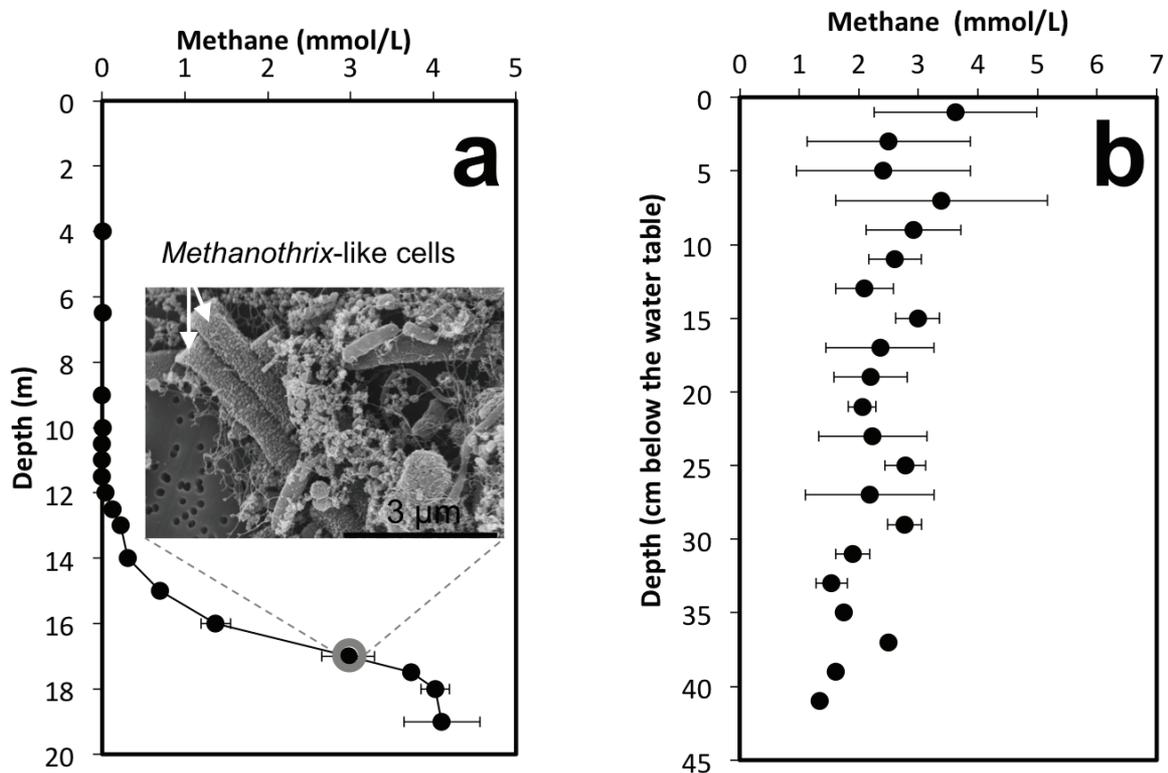


Figure 2. Methane profiling through the water column and sediment of lake La Cruz. (a) In the water column of Lake La Cruz, the highest methane concentrations were below 17-m depth where *Methanotherix*-like cells (inset) could be observed by scanning electron microscopy. (b) Sediment cores showed very high methane concentrations especially in the top 15-cm, indicating that methane has also sedimentary origin. The water column values as average \pm standard error values are for triplicate samples taken at each specific water column depth; while values for sediments are from triplicate cores sampled every 2-cm. *Perfiles de metano en la columna de agua y en el perfil de los sedimentos de la laguna de La Cruz. (a) En la columna de agua de la laguna de La Cruz, las concentraciones más altas de metano se encontraban por debajo de los 17 m de profundidad, donde células similares a Methanotherix (recuadro) se podían observar mediante microscopía electrónica de barrido. (b) Los testigos de sedimento mostraron concentraciones muy elevadas de metano, especialmente en los 15 cm superiores, lo que indica que el metano también tiene su origen en el sedimento. Los valores en la columna de agua son la media \pm el error estándar de muestras triplicadas tomadas a cada profundidad específica en la columna de agua; mientras que los valores en los sedimentos son la media de testigos triplicados muestreados cada 2 cm.*

cutoff. For taxonomy and diversity analyses, clean and clustered OTUs were analyzed using QIIME (Caporaso *et al.*, 2010), against the Ribosomal Database Project database version 11. Alpha rarefaction analyses showed sufficient coverage of the diversity in all three sediment cores.

DNA extractions from mud-free incubations were performed using the MasterPure DNA purification kit as previously described (Rotaru *et al.*, 2014b). Amplification of bacterial (27F, 5'-AGAGTTTGATCMTGGCTCAG and 1492R, 5'-TACCTTGTTACGACTT) and archaeal (344F – 5'-ACGGGGYGCAGCAGGCGCGA -3' and 1059R – 5'-GCCATGCACCWCCTCT-3') 16S rDNA sequences, library preparation, and 16S rRNA gene sequencing, was performed as previously described (Rotaru *et al.*, 2018). Maximum likelihood phylogenetic trees were constructed using Geneious (Kearse *et al.*, 2012). Sequence files can be found at NCBI under Bioproject ID: PRJNA510210.

RESULTS AND DISCUSSION

Our hypothesis was that the iron-rich Lake La Cruz would be a breeding ground for conductive, mineral-based syntrophy (Rotaru *et al.*, 2018). We discovered that microorganisms enriched from Lake La Cruz carried out syntrophic degradation strictly dependent on conductive mineral additions and were unable to carry unaided DIET associations.

Geochemistry

We expected to find a niche for DIET/conductive-particle mediated IET in this Fe-rich methanogenic lake resembling the ocean in the Precambrian. La Cruz sediments displayed high methane concentrations in the top 15 cm, along with a significant proportion of reactive iron species (~70 % of the total Fe content) (Thompson 2018), which is very high relative to normal non-ferruginous aquatic environments (Poulton & Raiswell, 2002). During this sampling campaign, the sediments were overlain by ~10 m of anoxic water (Fig. 1). During summer months, the lake is known to persistently have a 4-5 m monimolimnion zone above the sediment, which is rich in Fe²⁺ (Vicente & Miracle,

1988). In our study we also noticed strong methane super-saturation near the bottom, where the methane concentration reached 4 mM, similar to concentrations in the surface sediment (Fig. 2). Gas ebullition was observed during sampling from the deep water table. Oftentimes, gas bubbles mainly consisting of methane and carbon dioxide (Camacho *et al.*, 2017), percolated through the surface of the lake from the middle, as documented by an ecogram of the lake (Fig. 1d). Previous studies suggest that the sediment is the source of water-column methane (Oswald *et al.*, 2016). Indeed we observed that sediment methane concentrations were highest in the top centimeters of the sediment (Fig. 2). Methane concentrations were also high in the water column (17-20 m), indicating methanogenesis occurred in the bottom waters as well as the top layers of the sediment (Fig. 2).

As documented previously (Oswald *et al.*, 2016; Camacho *et al.*, 2017), dissolved Fe²⁺ did build up below the chemocline to reach concentrations of ~250 μM above the sediment-water interface. In the sediment, dissolved Fe²⁺ concentrations continued to increase (Thompson, 2018), reaching a peak of > 1000 μM at a depth of ~22 cm. These high dissolved Fe²⁺ concentrations in the water column and sediment porewaters are similar to those found in other iron-rich lakes (Vicente & Miracle, 1988; Bura-Nakic *et al.*, 2009; Crowe *et al.*, 2011; Nordi *et al.*, 2013). The La Cruz sediments were high in TOC (average = 6.68±2.0 wt %), and carbonate minerals (average = 9.46±1.3 wt % inorganic C), which diluted the total Fe-content to 1.06±0.18 wt % on average (Thompson, 2018). This is considerably lower than the average global total Fe content of riverine particulates supplied to oceans and lakes (4.49 wt %; Poulton & Raiswell, 2002). Proportionally, however 'reactive' Fe phases (non-sulphidized Fe²⁺, Fe-oxides, Fe-sulfides) were abundant (70±8 %; Thompson, 2018) relative to the total Fe content of the sediment, of which only 18±5 % was sulfur bound (pyrite, other Fe-S minerals). Nevertheless, magnetite Fe accounted for less than 0.1 % of the total Fe-content in this sediment, while other Fe oxide minerals accounted for ~10 % of total Fe on average. Thus, non-sulphidized particulate Fe(II) was the dominant reactive Fe pool (~60 % on average).

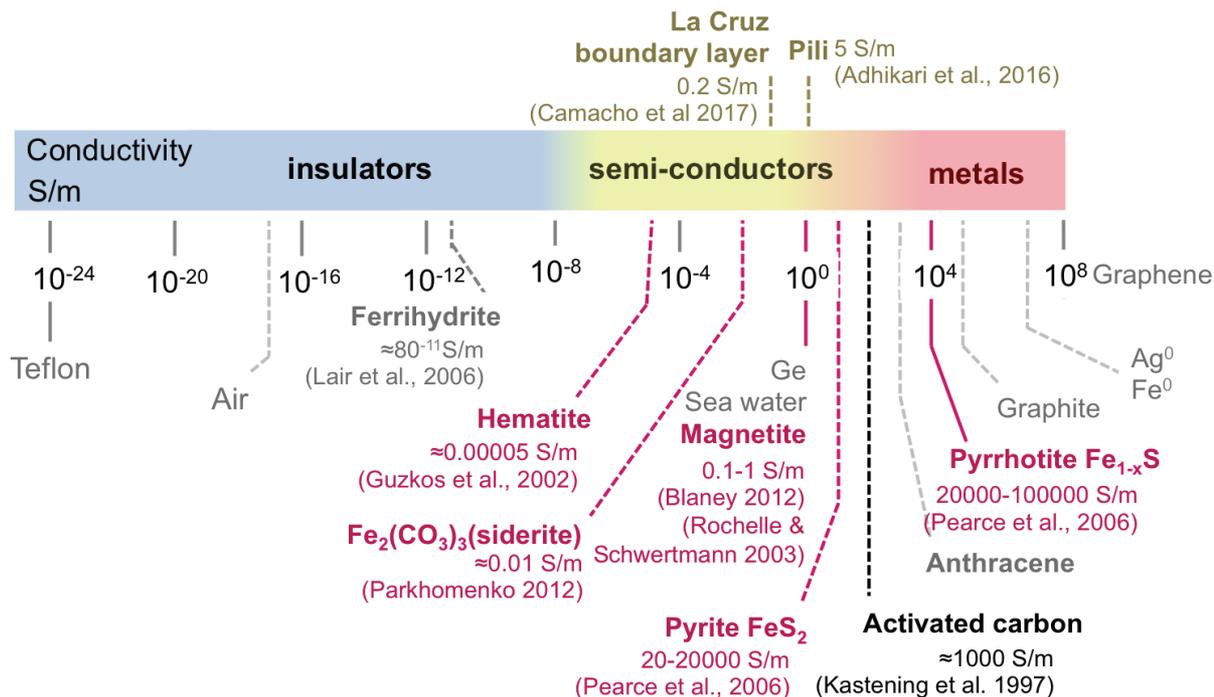


Figure 3. Conductivities of various Fe-oxides and Fe-sulfides, compared to that of the e-pili and the conductivities observed for different carbon particles including activated carbon used in this study. We have also listed the conductivity measured in lake La Cruz at the sediment-water boundary layer. (Adhikari *et al.*, 2016; Blaney, 2012; Camacho *et al.*, 2017; Guskos *et al.*, 2002; Kastening *et al.*, 1997; Lair *et al.*, 2006; Parkhomenko, 1990; Pearce *et al.*, 2006; Rochelle and Schwertmann, 2003). *Conductividades de varios óxidos y sulfuros de Fe, comparadas con las de los pelos bacterianos conductores (e-pili) y las conductividades observadas para diferentes partículas de carbón incluyendo el carbón activado utilizado en este estudio. También hemos enumerado la conductividad medida en la laguna de La Cruz en la interfase sedimento-agua.* (Adhikari *et al.*, 2016; Blaney, 2012; Camacho *et al.*, 2017; Guskos *et al.*, 2002; Kastening *et al.*, 1997; Lair *et al.*, 2006; Parkhomenko, 1990; Pearce *et al.*, 2006; Rochelle & Schwertmann, 2003).

Some of the iron minerals (Fe-sulfides and Fe-oxides) found in the sediments of lake La Cruz are electrically conductive (Fig. 3), of which magnetite and iron sulfides have been documented to facilitate mineral mediated syntrophy (Liu *et al.*, 2012, 2015; Zheng *et al.*, 2017; Rotaru *et al.*, 2018; Kato & Igarashi, 2018). Fe-sulfides, like pyrite have been also shown to aid long-range extracellular electron transfer from cells (Kondo *et al.*, 2015) or enzymes (Mahadevan & Fernando, 2018) to electrodes. Moreover La Cruz sediments also contain coal particles (Romero-Viana *et al.*, 2011), which are conductive (Fig. 3). Indeed it has been documented that conductive carbon materials (i.e. granular activated carbon) facilitated mineral mediated syntrophy as effectively as conductive Fe-minerals (Liu *et al.*, 2012; Rotaru *et al.*, 2018).

***In situ* bacterial diversity – with focus on described electrogens**

We therefore anticipated that electrically conductive particles inherent to La Cruz sediments would facilitate mineral mediated interactions between electrogens and electro-trophic methanogens. Indeed, our data demonstrate that the community harbors organisms affiliated to groups of electrogens including *Geobacter* (Fig. 4), and to DIET-methanogens including *Methanotherix* (Fig. 4). *Geobacter* and *Methanotherix* have previously been shown to carry out DIET in laboratory co-cultures (Rotaru *et al.*, 2014a), and have been found to co-exist in several man-made environments, such as rice paddies (Holmes *et al.*, 2017) and anaerobic digesters (Morita *et al.*, 2011; Rotaru *et al.*, 2014a). Our data showed that bacte-

ria affiliated to known electrogens/iron-reducers like *Geobacter* (0.6 % of all Bacteria), *Thiobacillus* (0.2 % of all Bacteria), *Desulfobacterium* (0.4 % of all Bacteria), and *Anaerolinea* (0.1 % of all Bacteria) co-existed with *Methanothrix* in Lake La Cruz sediments (Fig. 4). Together, all of these putative electrogens/iron reducers, summed up to circa 1 % of all Bacteria. Previously, members of these four genera, *Geobacter*, *Thiobacillus*, *Desulfobacterium*, *Anaerolinea*, have been shown to be capable of extracellular electron transfer to and/or from electrodes or metallic iron (Nakasono *et al.*, 1997; Dinh *et al.*, 2004; Gregory *et al.*, 2004; Kawaichi *et al.*, 2018; Pous *et al.*, 2014; Rotaru *et al.*, 2015), as well as iron-minerals (Bosch *et al.*, 2012; Kawaichi *et al.*, 2013; Lovley *et al.*, 1993; Rotaru *et al.*, 2015). The first two, *Geobacter* and *Thiobacillus* can also interact

by DIET with other cells (Summers *et al.*, 2010; Kato *et al.*, 2012; Rotaru *et al.*, 2014b, 2014a;), and this interaction has been shown to be expedited in the presence of conductive particles (Kato *et al.*, 2012; Liu *et al.*, 2012; Chen *et al.*, 2014; Liu *et al.*, 2015; Rotaru *et al.*, 2014b; Zheng *et al.*, 2017). It is therefore possible that all of these electrogenic species compete for the electron uptake of electrogenic methanogens.

However, one of the most abundant genera in these sediments was *Smithella* (2.6 % of all bacteria), which has been assumed to be electroactive and carry DIET interactions with *Methanothrix* in an alkane-degrading consortium (Embree *et al.*, 2014). Therefore, *Smithella* may establish a DIET-association with *Methanothrix* from Lake La Cruz sediments (see Archaea community below). Members of *Verrucomicrobia*

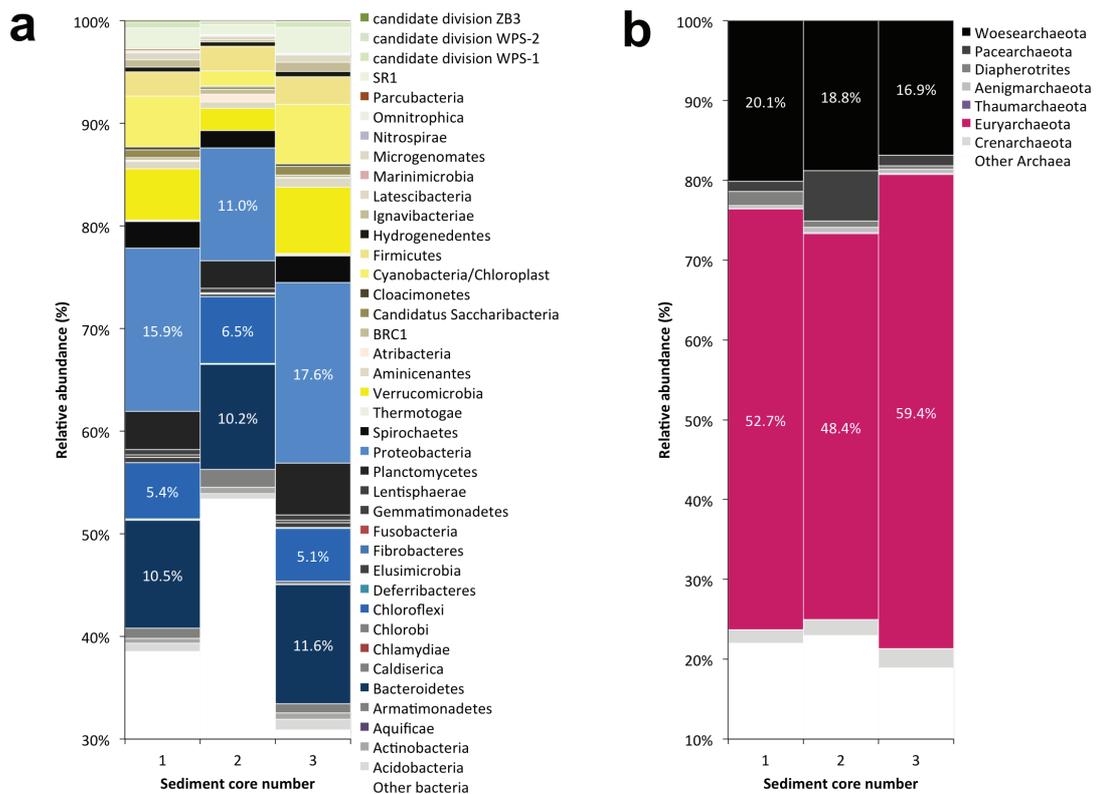


Figure 4. Relative phylum-level composition of (a) Bacteria and (b) Archaea harboring the top 16-cm of three sediment cores from lake La Cruz, as determined by 16S rRNA gene amplicon sequencing. *Composición relativa a nivel de filum de (a) Bacterias y (b) Arqueas albergadas en los 16 cm superiores de tres testigos sedimentarios de la laguna de La Cruz, según lo determinado por la secuencia del amplicón del gen 16S rRNA.*

were also very well represented (ca. 4.6 % of all bacteria) similar to what has been observed for 90 % of several lake sediments (He *et al.*, 2017). *Verrucomicrobia* were recently proposed to carry extracellular electron transfer due to their genetic make-up, which comprises the appropriate porin systems and membrane-associated *c*-type cytochromes (He *et al.*, 2017). It is also possible that *Verrucomicrobia* play a role in mineral mediated interspecies interactions. Nevertheless, *Verrucomicrobia* have never been shown to have the ability to interact syntrophically or to carry out extracellular electron transfer in laboratory cultures, and thus this predicted physiology requires further investigation. Some of the most abundant phyla were Bacteroidetes (10.8 % of the bacteria), and Firmicutes (2.5 % of all bacteria) (Fig. 4).

***In situ* archaeal diversity**

Euryarchaeaota accounted for more than half of the *Archaea* represented through amplicon sequencing (Fig. 4). Here, we show that in the sediments of Lake La Cruz, *Methanothrix* co-existed with electrogens (*Geobacter*, *Thiobacillus*, *Desulfobacterium*, and *Smithella*). Besides the acetoclastic/DIET-associated *Methanothrix* (3.7 % of all archaea), we identified canonical hydrogenotrophic-methanogens belonging to *Methanoregula* (2.5 % of all archaea), and very low numbers of *Methanobacterium* (0.2 % of all *Archaea*). The most abundant *Archaea* were the deep-branching *Methanomassilicoccus* (40.6 % of all archaea). The role of *Methanomassilicoccus* in sedimentary methanogenesis is not well understood since their only cultivated species-representative, *M. luminyiensis*, is a human-gut isolate strictly capable of H₂-dependent methylotrophic methanogenesis, but incapable of CO₂-reductive methanogenesis or acetoclastic methanogenesis (Dridi *et al.*, 2012a). Besides their documented presence in the gut of humans (Dridi *et al.*, 2012b), other animals (i.e. Salgado-Flores *et al.*, 2016) or insects (Paul *et al.*, 2012), *Methanomassilicoccus* sp. have also been found in anaerobic digesters (e.g. Chojnacka *et al.*, 2015), hydrothermal springs (e.g. Coman *et al.*, 2013), subsurface aquifers and soils (e.g. Kadnikov *et al.*, 2017), and

aquatic sediments (e.g. Rotaru *et al.*, 2018) to name a few. *Methanomassilicoccus* was also one of the most abundant genera of methanogens, not only in the iron-rich sediments of Lake La Cruz, but also in Baltic Sea sediments that are potential niches for conductive particle-mediated syntrophy (Rotaru *et al.*, 2018). It is possible that *Methanomassilicoccus* is involved in electroactive interactions via minerals, especially taking into account that this group was recently associated with electroactive communities abundant on electrodes from bioelectrochemical systems set up with inoculums from soils (Ahn *et al.*, 2014) and anaerobic digester sludge (Park *et al.*, 2018).

Among the methanogens detected in La Cruz sediments, only species of *Methanothrix* have been previously shown to establish DIET-associations with *Geobacter* species (Morita *et al.*, 2011; Rotaru *et al.*, 2014a; Wang *et al.*, 2016; Holmes *et al.*, 2017). *Methanothrix* was earlier suggested to carry out DIET with *Smithella* (Embree *et al.*, 2014), but the latter has never been shown to be capable of mineral-mediated or direct electron transfer. In a previous study, we have shown that a *Methanothrix*-species from the Baltic did not establish a mineral-mediated interaction with Baltic-*Geobacter*, but were instead competitively excluded by a *Methanosarcina-Geobacter* consortium, which carried a mineral-mediated syntrophic association (Rotaru *et al.*, 2018). However, although *Methanosarcina* is a very effective DIET partner (Rotaru *et al.*, 2014b, 2015) and mineral-syntrophy partner (Liu *et al.*, 2012; Chen *et al.*, 2014; Wang *et al.*, 2018; Rotaru *et al.*, 2018) they were poorly represented in La Cruz sediments (Fig. 4).

High methanogenic activity could only be maintained by conductive particles

To determine the effect of conductive particles on the Lake La Cruz methanogenic community, we compared incubations with or without additional conductive particles. These incubations showed that the methanogenic community was strictly dependent on the addition of conductive particles and independent of the type of substrate, conductive particle, or freshwater medium tested (Fig. 5). Incubations with conductive particles showed 2 to

4 fold increases in methanogenic rates (0.2-0.7 mM/day, depending on substrate) over incubations with non-conductive glass beads or without particle-amendment (0.09 to 0.18 mM/day, depending on the substrate). Moreover, high methanogenic activity was maintained in subsequent incubations *only if* conductive particles were added (Fig. 5). Cultures without conductive particles could not sustain methanogenesis for more than one subsequent transfer. This indicates a strict dependency of the enriched methanogenic community on conductive particles.

We observed that all tested substrates were transiently converted to acetate, which was converted quickly to methane in the presence of conductive particles, whereas acetate accumulated in the absence of conductive particles (Fig. 5). This is likely due to higher rates of acetate oxidation prompted by the addition of conductive particles, similar to previous observations of Bothnian Bay sediments where syntrophic acetate oxidation (SAO) relied on conductive minerals (Rotaru *et al.*, 2018).

We determined which organisms were

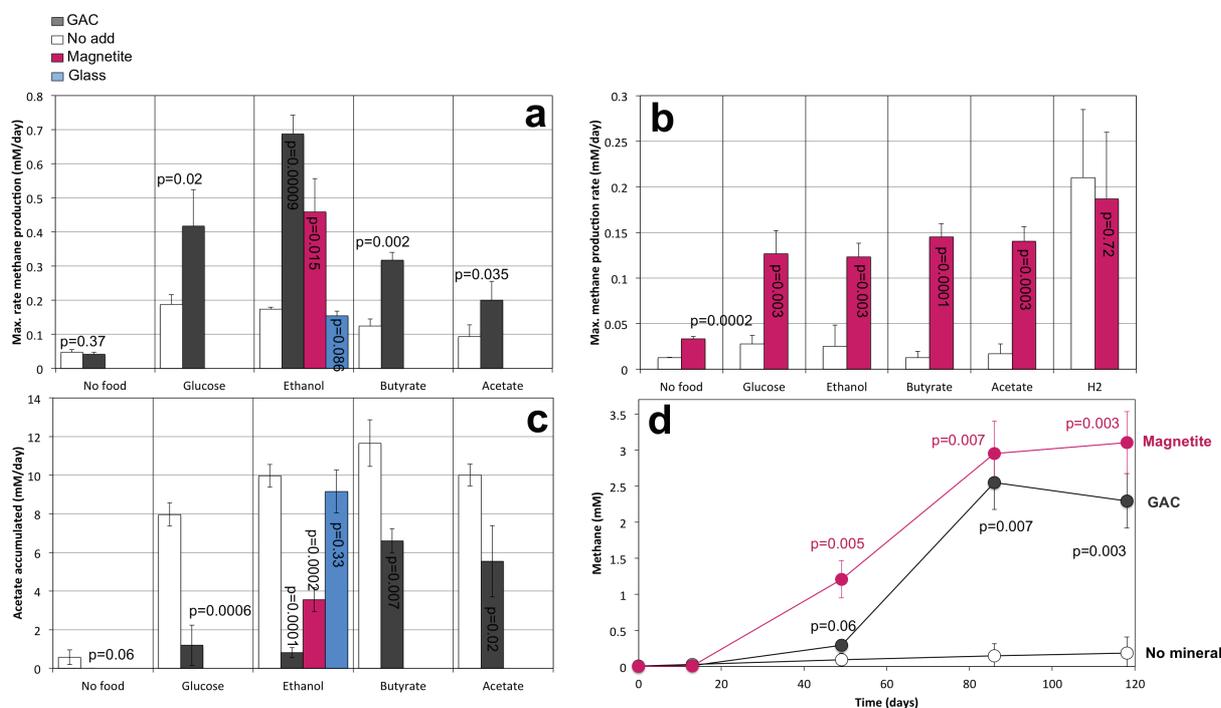


Figure 5. Methanogenesis on different substrates in incubations from lake La Cruz samples. Methane production in initial slurry incubations provided with different substrates was stimulated by conductive particles (GAC or magnetite) independent of the media used (a) modified DSMZ 120 or (b) DSMZ 334. (c) Acetate accumulated in incubations without conductive particles, but was significantly lower at the addition of conductive particles. (d) For example, a third transfer free of sediment showed that methanogenesis and acetate consumption were strictly dependent of the presence of conductive particles (colored symbols), and ceased if conductive particles were not added consistently for subsequent transfers (white symbols). Values represent average \pm standard deviation and are based on triplicate incubations, with the exception of the incubation with acetate plus GAC, where we had duplicates. *Metanogénesis con diferentes sustratos en incubaciones con muestras del lago La Cruz. La producción de metano en las incubaciones iniciales de lodos provistos con diferentes sustratos fue estimulada por partículas conductivas (GAC o magnetita) independientemente del medio utilizado (a) DSMZ 120 modificado o (b) DSMZ 334. (c) Acetato acumulado en incubaciones sin partículas conductivas, que fue significativamente menor tras la adición de partículas conductivas. (d) Por ejemplo, una tercera transferencia libre de sedimento mostró que la metanogénesis y el consumo de acetato dependían estrictamente de la presencia de partículas conductivas (símbolos de color), y cesaban si las partículas conductivas no se añadían consistentemente para las transferencias subsiguientes (símbolos blancos). Los valores representan la media y la desviación estándar y están basados en incubaciones triplicadas, excepto la incubación con acetato incorporado de GAC, que fueron por duplicado.*

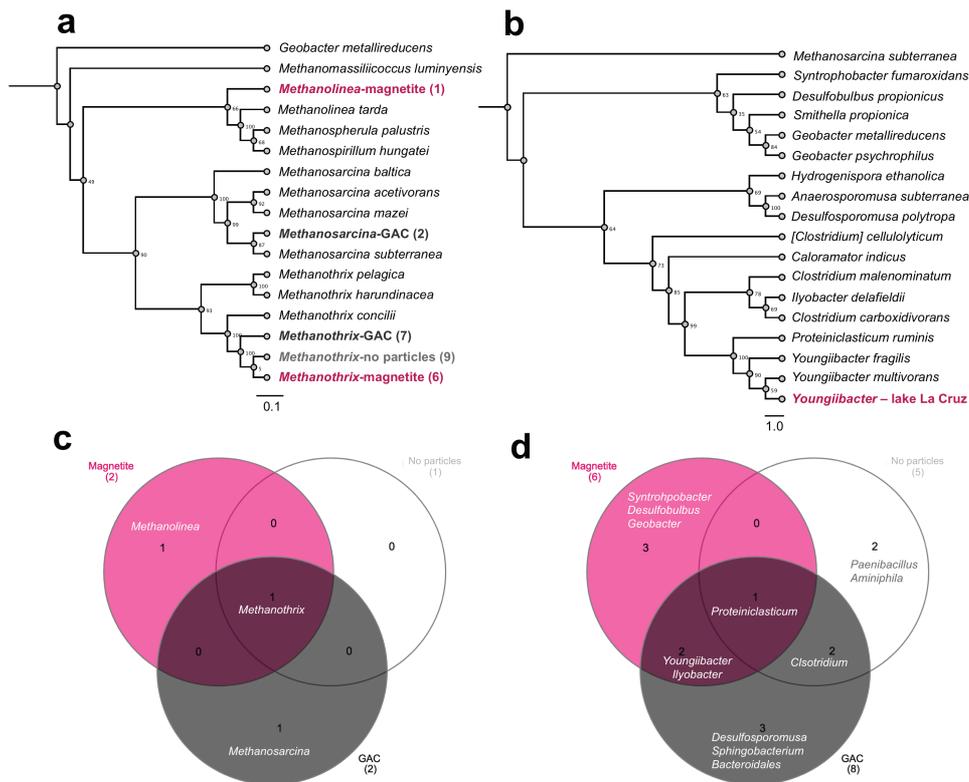


Figure 6. Maximum likelihood phylogenetic trees (a, b) and Venn diagrams with the relative distribution of 16S rRNA-gene sequences for Archaea (a, c) and Bacteria (b, d) in La Cruz incubations with or without conductive particles. Archaeal (a) and Bacterial (b) 16S rRNA genes were retrieved from third mud-free transfer of acetate-incubations with magnetite (pink), and GAC (black-bold) or from a first mud-free transfer without conductive particles (light gray-white). (c) The only Archaeal 16S phylotype encountered in all incubations independent of treatment was *Methanotherix*-related. (d) The most abundant Bacterial phylotype encountered only in conductive particle-amended incubations was *Youngiibacter*-related. *Árboles filogenéticos de máxima verosimilitud (a, b) y diagramas de Venn con la distribución relativa de las secuencias del gen 16S rRNA para Archaea (a, c) y Bacteria (b, d) en incubaciones de muestras de La Cruz con o sin partículas conductoras. Los genes Archaeal (a) y Bacterial (b) 16S rRNA se recuperaron de la tercera transferencia sin sedimento en incubaciones con acetato y magnetita (rosa) y GAC (negro fuerte) o de una primera transferencia sin sedimento y sin partículas conductoras (gris claro-blanco). (c) El único filotipo de 16S de Archaeas encontrado en todas las incubaciones, independiente del tratamiento, estaba relacionado con Methanotherix. (d) El filotipo bacteriano más abundante encontrado solo en incubaciones complementadas con adición de partículas conductoras fue del tipo relacionado con Youngiibacter.*

enriched on acetate with or without conductive particles. For this we compared the acetate fed communities exposed to two types of conductive particles (GAC and magnetite) to a community exposed to no conductive particles. We determined that *Youngiibacter* and *Methanotherix* methanogens dominated the enrichments amended with both types of conductive particle (Fig. 6). On the other hand, in controls without conductive particles, after only one single transfer *Youngiibacter* could not be detected. In the absence of conductive particles methane production only occurred slowly for

one transfer and in this case *Methanotherix* co-existed with *Clostridium* (Fig. 6).

Youngiibacter was only found in enrichments with conductive particles and its presence could be associated with rapid acetate consumption coupled to methane production (Fig 5). We therefore anticipate that *Youngiibacter* plays a role in conductive-particle mediated syntrophy. Nevertheless, until now little is known about this group of Firmicutes, and only recently two species of *Youngiibacter* have been described (Lawson *et al.*, 2014; Tanaka *et al.*, 1991), of which one is

associated with fermentation of organics on coal surfaces during coal conversion to natural gas (Lawson *et al.*, 2014). Coal, similar to activated carbon, is electrically conductive (Duba, 1977). Moreover, *Methanotrix* have been also found associated with coal conversion to natural gas (Beckmann *et al.*, 2011; Lawson *et al.*, 2014). It is therefore possible that *Youngiibacter* and *Methanotrix* play a role in conductive particle-mediated syntrophy in coal beds, and as well in Lake La Cruz sediments. However, a syntrophic association between *Youngiibacter* and *Methanotrix* has not been described before. We suggest that *Youngiibacter* released electrons from

substrate/acetate oxidation onto conductive minerals that are then used as a source of electrons for *Methanotrix* in order to reduce CO_2 to methane. It is possible that *Youngiibacter* releases electrons extracellularly using a similar mechanism to that described for *Geobacter* namely a network of outer membrane *c*-type cytochromes (OMC) and pili (Shrestha *et al.*, 2013). During DIET, OMCs were not as necessary for a donor *Geobacter* strain to carry substrate oxidation coupled with extracellular electron transfer (EET) and respiration, since OMCs could be completely replaced by the conductive iron oxide, magnetite (Liu *et al.*,

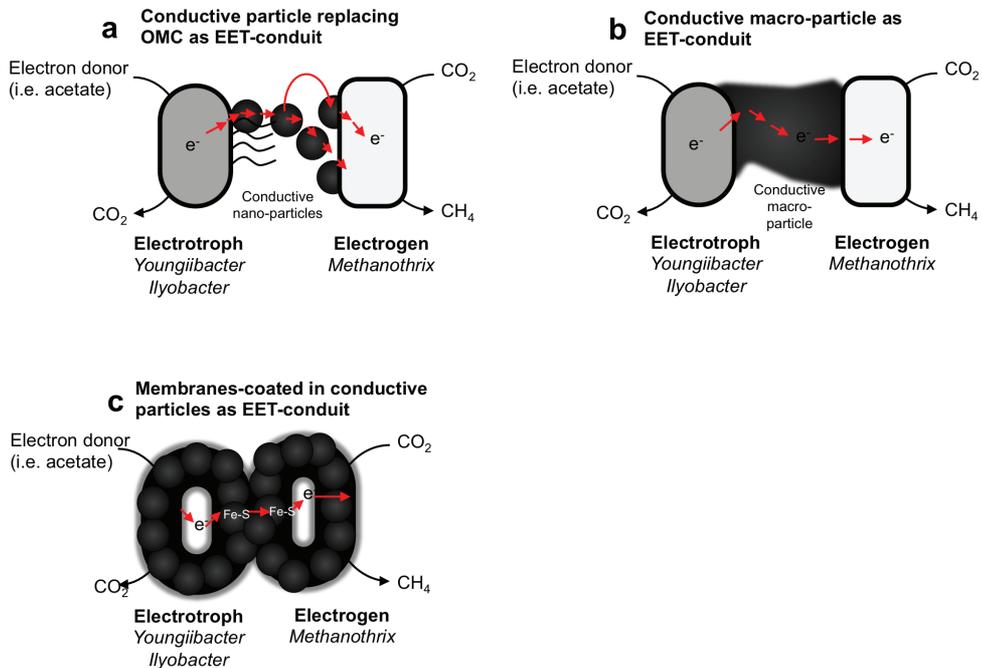


Figure 7. Proposed model interspecies interactions in La Cruz sediments facilitated by conductive particles. (a) Syntrophy mediated by a conductive nano-particles replacing outer membrane cytochromes (OMCs). Nevertheless, pili involved in EET are still available. (b) Syntrophy mediated by a conductive macro-particle (e.g. GAC), which plays the role of both and electron plug and outlet. (c) Syntrophy mediated by a conductive-mineral coat padding the cell surface. In lake La Cruz, conductive minerals could for example result from the precipitation of Fe^{2+} as Fe-S/thiol-. Cell surfaces encrusted with a Fe-S coat might endorse the electron-transfer between the two distinct metabolic entities even in the absence of a typical EET/DIET conduit. *Modelo de interacciones interespecies propuesto para los sedimentos de La Cruz facilitados mediante partículas conductivas. (a) Sintrofia mediada por nanopartículas conductivas que reemplazan a los citocromos de membrana externa (OMC). Sin embargo, los pili involucrados en EET todavía están disponibles. (b) Sintrofia mediada por una macropartícula conductiva (es decir, GAC), que desempeña el papel de ambos, la conexión y la fuente de electrones. (c) Sintrofia mediada por una envoltura mineral conductiva que rellena la superficie celular. En el lago La Cruz, los minerales conductivos podrían, por ejemplo, resultar de la precipitación de Fe^{2+} como Fe-S/tiolatos- en el espacio periplásmico de las células. Las superficies celulares incrustadas con una envoltura de sulfuros metálicos como Fe-S podrían avalar la transferencia de electrones entre las dos entidades metabólicas distintas, incluso en ausencia de un típico conducto EET / DIET.*

2015). Instead, when it plays the role of electron donor *Geobacter* seems to necessitate e-pili for long range electron transfer to partner cells, as exemplified in a recent study (Ueki *et al.*, 2018). In agreement with previous observations in *Geobacter*, *Youngiibacter* might employ type IV pili for EET to partner *Methanotherix*. *Youngiibacter*'s type IV pili gene sequence (T472_0202395) differs greatly from that of *Geobacter* in length (238 aminoacids longer than its correspondent in *G. metallireducens*) and sequence identity (only a stretch of 16 aminoacids showed 50 % identity). Nevertheless, this prepilin-gene encodes for a protein with high content of aromatic aminoacids (10.3 %) which could give this organism an advantage to carry EET (Walker *et al.*, 2018). It is possible that conductive particles ornate the pili of *Youngiibacter* in a similar way to how they do for *Geobacter* (Liu *et al.*, 2015; Wang *et al.*, 2018) facilitating electron transfer to syntrophic partner methanogens.

Conductive-particle mediated syntrophy

Syntrophy mediated by conductive particles could occur in three different ways (Fig. 7). A first mode of action includes electrogens with limited expression of surface cytochromes whose role would be replaced with that of conductive minerals (pyrite, pyrrhotite, magnetite, goethite) found in sediments (Fig. 7a). Molecular and microscopic evidence for this type of association has been brought by studies in laboratory *Geobacter* co-cultures provided with magnetite (Liu *et al.*, 2015). A second possibility is that cells plug into macro-sized conductive rocks (i.e. iron/manganese-nodules) with one cell releasing electrons onto the rock and the other receiving electrons (Fig. 7b). Evidence for such interactions was previously obtained in laboratory co-cultures with macro-sized conductive chars. In this case, using SEM, it was shown that the electrogen/*Geobacter* did not require direct contact to the electrotroph/*Methanosarcina* yet the conductive surface facilitated the syntrophic association (Chen *et al.*, 2014; Liu *et al.*, 2012). The third possibility (Fig. 7c), is that membrane-bound proteins facilitate the precipitation of Fe²⁺-ions,

e.g., with thiol groups (Milner-White & Russell, 2005) to form a conductive surface-conduit surrounding the cell. Extracellular electron transfer between such mineral-coated cells has been proposed (Kato *et al.*, 2012), but has not been confirmed. However, this could be a possibility for microbes without an extracellular apparatus for electron transfer to partner cells.

In evolutionary terms, it is plausible that mineral-mediated interactions preceded interspecies electron transfer interactions based in diffusible chemicals, which require complex enzymes and cell-bound electrical conduits. Primordial protocells had not developed enzymatic machineries to maintain redox and proton gradients across cell membranes (Martin *et al.*, 2003; Russell *et al.*, 1990, 1994; Wächtershäuser, 1988b). It has therefore been suggested that minerals, which can uphold voltage differences, such as FeS/pyrite, might have helped nucleate the earliest membranes, playing the role of early membrane-bound catalysts, instead of electron transport chain enzymes (Martin *et al.*, 2003). Later, the high reactive iron content of the Archaean ocean could have promoted the formation of proteins with Fe-S centers which are required and abundant in redox proteins of methanogens (Liu *et al.*, 2010). Here, we propose that primitive cells with leaky membranes (Lane & Martin, 2012), allowed easy electron transfer via conductive minerals permitting energy exchange between separate metabolic protocell entities. Thus, conductive particles could have fostered the earliest interspecies interactions in the methanogenic and iron-rich Early Earth oceans, and possibly nurtured adaptation of interspecies associations pre-eukaryogenesis.

CONCLUSION

In conclusion, we show that the sediment of an early Earth ocean analogue is a niche for syntrophic associations dependent on conductive particles. *Only if* conductive particles were provided, could syntrophic bacteria coupled to methanogens oxidize their substrates. Thus, only in incubations with conductive particles, members of the genus *Youngiibacter* co-existed with *Methanotherix*. Incubations without conductive

particles resulted in the disappearance of *Youngiibacter*, and one transfer later to the demise of the methanogenic community. These data indicate that conductive particles were required to aid the pairing of the metabolism of *Youngiibacter* with that of *Methanothrix*, which sustained high rates of methanogenesis in this early Earth analogue – lake La Cruz. We propose that obligate mineral-syntrophy is an ancestral interspecies interaction established before complex membrane structures and enzymes evolved to mediate direct or indirect associations between species with distinct metabolism.

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Con el apoyo de:

