Data Repository File DR1: Supplementary Information

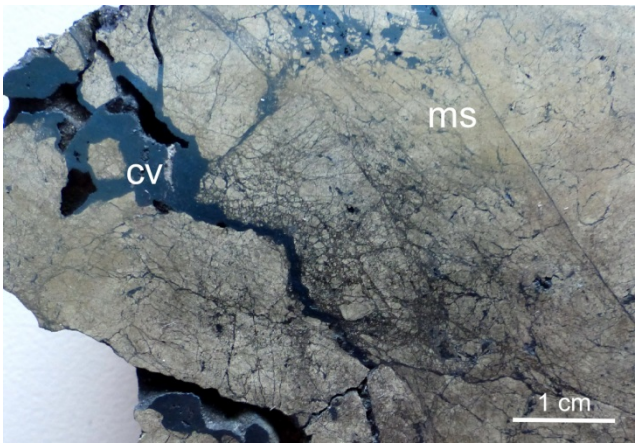

Figure DR1. Brecciated fine-grained primary massive sulfides (ms) rich in pyrite with fractures infilled by massive covellite (cv). Sample LC-4.

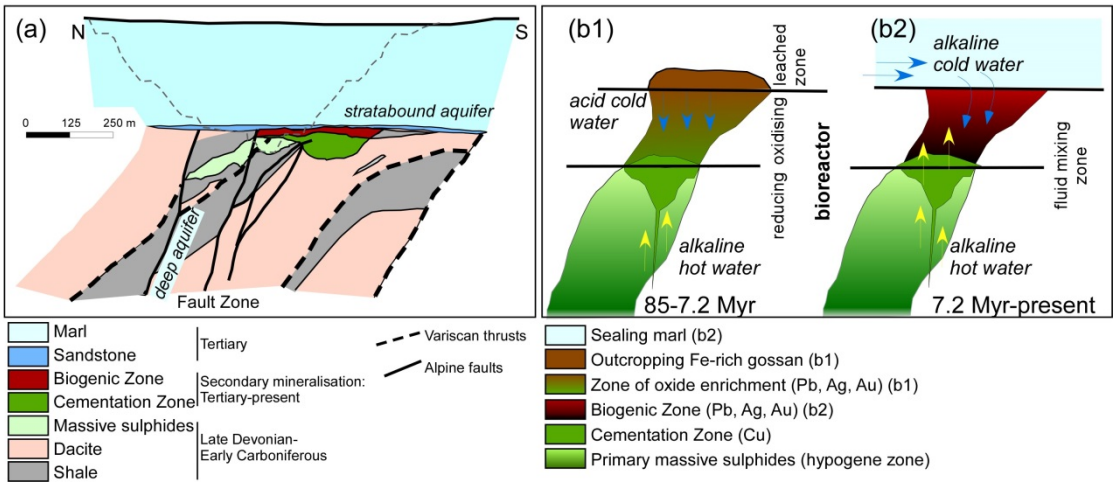

Figure DR2. (a) North-south geological cross section of Las Cruces deposit showing current location of cementation zone beneath the biogenically modified gossan and thick cover of unconformable marl sequence. (b) Idealized cross section showing proposed evolution of secondary mineralizing processes at Las Cruces, including first stage formed under subaerial conditions (b1) followed by a younger stage developed below thick sedimentary cover (b2). Modified from Tornos et al. (2017).

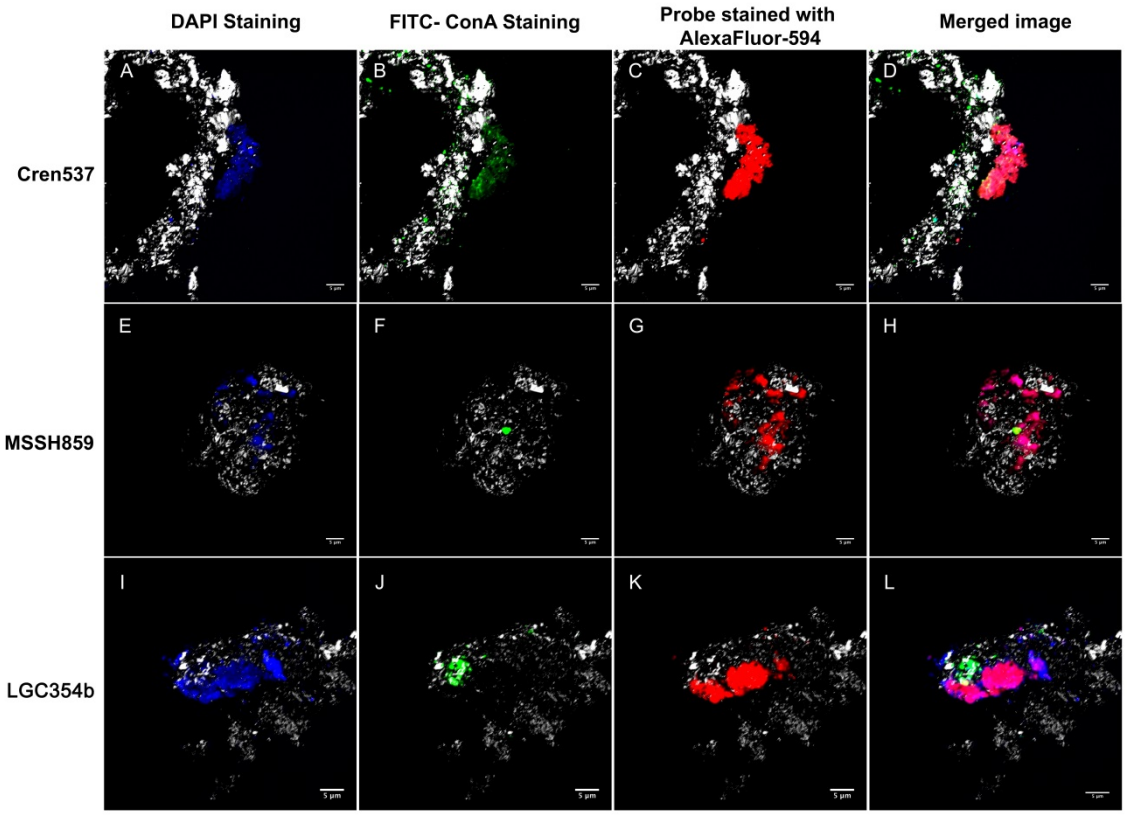

Figure DR3. Representative micrographs of hybridized subsurface environmental samples from Las Cruces mine analyzed by CARD-FISH using different oligonucleotide probes. Blue signal (A, E, and I) shows microbial DNA stained with DAPI. Gray and white signal shows host sulfide minerals (predominantly covellite) in all cases. B, F, and J (green) show glucose/mannose residues of microbial EPS stained with FITC-ConA. Red signal (AlexaFluor-594-Tyr) corresponds to hybridization signals obtained by different oligonucleotide probes. (C) Crenarchaeota phylum members with Cren537 probe; (G) Methanogenic archaea from Methanosarcinales order are hybridized with MSSH859 probe; (K) Firmicutes targeted with LGC354b probe; D, H, and L show merged image of three previous channels (blue, green, red). Scale bars represent 5 µm in all cases.

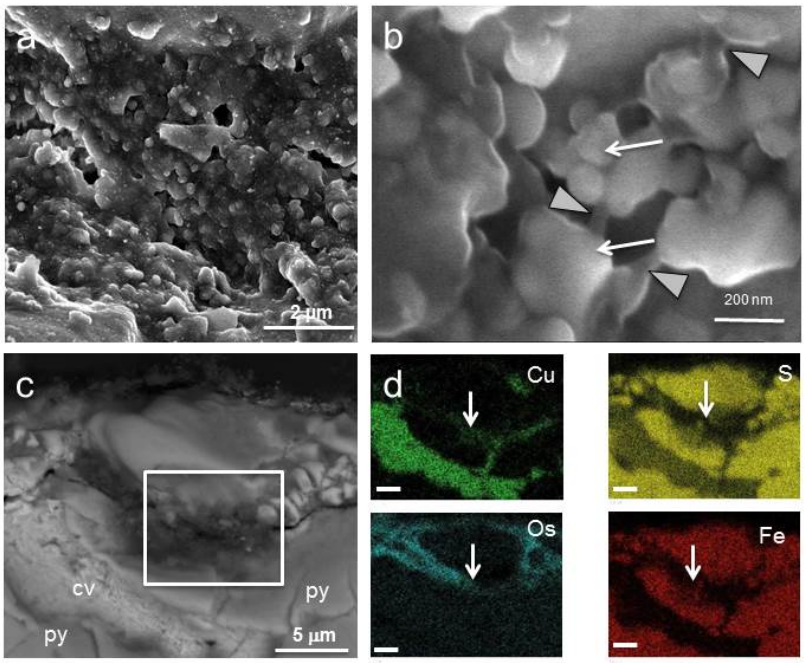

Figure DR4. SEM images of fractures in massive sulfides infilled with covellite and hosting microorganisms, related EPS, and currently precipitating covellite. Sample LC-5. (a) FE-SEM image showing microbial cells infilling cavities within veins of massive covellite. (b) FE-SEM image showing EPS (arrowheads) associated with microbial cells (arrows) within endolithic microbial aggregates; these cells have sizes and morphologies similar to those observed by CARD-FISH (Fig. 2). (c) SEM-BSE image of area in Fig. S3D (marked by square). (d) EDS map of copper (Cu), sulfur (S), osmium (Os), and iron (Fe) contents in area shown in Fig. S3C. Fe and S mark zones dominated by hosting pyrite, Cu and S show presence of secondary covellite, and Os denotes presence of microbial cells and EPS; zones with superimposed Cu, S, and Os signals define areas where neoformed covellite crystals are associated with living microbial cells (Fig. 3).

Methods

Rocks from the cementation zone at Las Cruces were extracted from a subvertical drill core (CR503) sited at the bottom of the open pit at a depth of ca. 160 m below the pre-mining surface (collar coordinates 37°30'7.41"N; 6°51'32.43"W, 148.8 m bsl), using conventional exploration drilling techniques with a PQ core diameter (85 mm). In order to evaluate possible contamination, the water used for drilling was doped with NaBr (10 mg/l). All intervals of the drill core showing secondary sulfides were encapsulated in sealed plastic bags under an N_2 atmosphere soon after extraction. Some samples containing cm-sized fragments of pyrite-rich massive sulfide cemented and supported by mm-thick veins of massive covellite (core depths of 12.20-12.50 m for LC-4 and 15.85-16.15 m for LC-5) were chosen for further analysis. Sealed sample bags were opened in a N_2-H_2 atmosphere and the rocks were split into three or more fragments. One fragment was used to prepare polished thin sections for petrographic studies. Another was washed in distilled water and analyzed for Br in order to detect contamination by external fluids; significantly, no Br was detected in any sample. Finally, remaining fragments of the rock were broken into smaller pieces in an agate mortar under anaerobic conditions, and aliquots were used for microbiological studies, including:

For CARD-FISH (Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization) analysis, rock powders and small chips were fixed in the field soon after fragmentation in a 4% (v/v, final concentration) formaldehyde- and fosfate-buffered saline (PBS) (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4) solution at 4° C for 2 hr. Samples were washed twice with PBS and stored at -20° C in 1:1 Ethanol:PBS. Later, ca. 150 mg of rock was selected for analysis: the sample was embedded in 0.2% (w/v) agarose. Endogenous peroxidases were inactivated in 0.1% H_2O_2 in methanol for 30 min at room temperature. Hybridization was performed following the method described by Pernthaler et al. (2004) with some modifications in order to facilitate the handling of small fragments of rock. The fluorochrome used was AlexaFluor594 labeled tyramide. Samples were counterstained with ConcanavalinA labelled with fluorescein isocyanate (FITC-ConA) lectin from Canavalia ensiformis in order to reveal the presence of glucose and/or mannose in the extracellular polymeric substances (EPS) in biofilms (Poretz and Goldstein, 1970), due to its binding specificity to alpha-D-methyl-mannose and alpha-D-methyl-glucose moieties.

Lectin was prepared from stocks of 1 mg/ml in deionized water; the hybridized rock samples were incubated for 30 min in darkness at room temperature, and washed twice with deionized water (Raho et al., 2012). Subsequently, samples were stained with 4', 6'-diamidino-2-phenylindole (DAPI), which binds to DNA and is used to stain and visualize it. Different oligonucleotide probes were used for targeting rRNA genes, including: EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') (Amann et al., 1990) mixed with EUB338-III (5'-GCTGCCACCCGTAGGTGT-3') (Daims et al., 1999) for most bacteria at 35% (v/v) formamide (FA) concentration; ARC915 (5'-GTGCTCCCCCGCCAATTCCT-3') (Stahl and Amann, 1991) for archaea, at 20% (v/v) FA; GAM42a (5'- GCCTTCCCACATCGTTT-3') (Manz et al., 1992) for gamma-proteobacteria at 35% (v/v) FA; Cren537 (5'- TGACCACTTGAGGTGCTG-3') (Teira et al., 2004) for Crenarchaeota at 20% (v/v) FA; LGC354a (5'-TGGAAGATTCCCTACTGC-3') 54 and LGC354b (5'- CGGAAGATTCCCTACTGC-3') (Meier et al., 1999) for most Firmicutes at 35% (v/v) FA; DSP648 (5'-CTCTCCTGTCCTCAAGAT-3') (Gonzalez-Toril et al., 2006) for Desulfosporosinus,

Desulfitobacterium and Dehalobacter at 30% (v/v) FA; SRB385 (5'-GTTCCTCCAGATATCTACGG-3') (Amann et al., 1990) for sulfate-reducing bacteria at 35% (v/v) FA ; MSSH859 (5'-TCGCTTCACGGCTTCCCT-3') (Boetius et al., 2000) for Mathanosarcinales at 35% (v/v) FA, and ACI145 (5'-TTTCGCTTCGTT A TCCCC-3' (Schulze et al., 1999) for Acidovorax at 35% (v/v) FA. HRP-labeled probes were synthesised by Biomers.net GmbH (Ulm, Germany). Negative controls were performed with the control probe NON338 (5'-ACTCCTACGGGAGGCAGC-3') (Wallner et al., 1993), which does not hybridise with any microorganism, and would therefore indicate the nonspecificity of the probe with the rock sample. Additional negative controls were performed carrying out the entire CARD-FISH protocol but without any probe; the complex fluorophore-tyramide was evaluated if it could interact with some mineral. In both cases, the results were negative.

Estimation of the number of cells per rock unit were performed by vortexing fixed powder and small rock chips at 4° C for 15 minutes in a Sekar Buffer (0.05% Tween80, 150mM NaCl). Supernatant was filtered through membrane filters of 0.22 µm pore size (Millipore, USA) and washed with 1X PBS. Filters were embedded in 0.2% (w/v) agarose, and subjected to CARD-FISH procedure as described previously with mixed EUB338 and EUB338-III, ARC915, and NON338 probes. Counts were performed manually with a fluorescence microscope Axioskop (Zeiss) by triplicate and at least 15 counting grids were counted per membrane section.

For electronic microscope studies, samples were mounted onto ibidi µ-slides (ibidi GmbH, Germany) embedded in Citifluor:Vectashield (4:1), and were examined with a Nikon AiR+ Resonant Scanning Confocal System (Nikon) at the Confocal Microscopy Service at the CBMSO (Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid).

Observations under Field Emission-Scanning Electron Microscopy (FE-SEM, Philips XL30 S-FEG) were performed at the SIdi (Servicio Interdepartamental de Investigación, Universidad Autónoma de Madrid) and under Scanning Electron Microscope (SEM, JEOL JSM-5600 LV) combined with EDX at the Museo Nacional de Ciencias Naturales (CSIC, Madrid). Rock samples were fixed in the field in formaldehyde, as described above, and were then processed following the procedure developed for observing the rock-microorganism interface by SEM-Back Scattered Electron (SEM-BSE) mode (Wierzchos and Ascaso, 1994). This method includes the fixation of samples in glutaraldehyde and osmium tetroxide solutions, dehydration in a graded ethanol series, and embedding in LR-white resin; these blocks of resin-embedded rock samples were finely polished. OsO_4 is used as a tissue fixative that binds to lipidic compounds and consequently denotes the presence biological components. The samples were mounted onto conductive graphite stubs and carbon coated in a Bio-Rad SC 502 apparatus for electrical conductivity and for preventing charging under the electron beam. Qualitative element compositions were determined by Energy-Dispersive X-ray Spectroscopy (EDS) microanalysis using an INCAx-sight instrument with a Si-Li detector (Oxford, England; detection limit ca. 10% for major elements). This system is able to detect some light elements (C, O, and N); semi-quantitative estimations were obtained by referencing as defaults to the higher peaks obtained in each spectrum, which generally corresponded to carbon. Analyses were performed at room temperature using an accelerating voltage of 20 kV and working distance of 20 mm.

Enrichment cultures for the determination of the different metabolic activities were performed using the methodology described by Sanz et al. (2011). Experiments were carried out in 130 ml serum bottles sealed with butyl rubber stoppers. The bottles were autoclaved and then flushed with N_2:CO_2 80:20 for 3 min. Incubation was static, in the absence of light, and done in a thermostatic chamber at 30 ± 2° C. Cultures for methanogenic activity included three different substrates: acetate (Mg1), H_2 (Mg2), and a mixture of C3, C4, and methanol (Mg3). For acetogenic activity, the substrates include a mixture of ethanol, propionic, butyric, lactic, crotonic, and fumaric acids. Methanotrophic activity was cultured with 100% methane; sulfate-reducing activity was assessed with H_2/acetate (SBR1) and a mixture of glycerol, methanol, and lactic acid (SBR2). Controls were provided by a mineral media without substrate. Occluded gases were measured after leaving the minimal head space by bubbling with 100% N_2. The released gas, using N_2 as a carrier, was analysed by gas chromatography (Bruker 450-GC), equipped with a thermal conductivity detector for CO_2 and H_2 and flame ionization for CH_4.

Analysis of the microbial communities (DNA extraction, PCR amplification, 454 pyrosequencing) was done at the Laboratorio de Metanogénesis, Universidad Autónoma de Madrid. Samples of biomass were collected from the reactors after 45 days of culture, then analysed in parallel with the inoculum. In order to minimise heterogeneity of the reactor biomass, three sub-samples were pooled. Total DNA extraction was performed using the FastDNA Spin kit for soil BIO101 (MPBio). Invitrogen Platinum Taq DNA polymerase and the primer sets 27F and 907R, labelled with three different barcodes, were used for the PCR reactions (program: 3' at 95°C followed by 28 cycles of 30" at 95° C, 45" at 54° C, and 90" at 68° C, plus a final extension step of 10' at 68° C). PCR products were purified with the Invitrogen Purelink kit. Library quantification was done by the fluorometry method using the Quant-iT PicoGreen dsDNA Assay Kit. A composite pyrosequencing sample was prepared by means of pooling approximately equal amounts of PCR amplicons from each sample. Pyrosequencing was performed by the Centro de Investigación Tecnológica e Innovación (University of Sevilla) using a 454 FLX+ System (Roche). All processing of sequences was done using the Mothur package v.1.36.0 (www.mothur.org; Schloss et al., 2009). Scripts were encoded to (1) remove any sequences containing more than one ambiguous base ('N'), (2) check the completeness of the barcodes and of the adaptors, (3) remove any sequences shorter than 150 bp, and (4) remove any sequences including those having low-quality base scores (Phred quality scores <25). Sequences were aligned with the SILVA 16S rRNA alignment Database Release 123 (www.arb-silva.de). Sequencing noises were removed with the Precluster tool of the Mothur package; chimeras introduced by the PCR process were detected and removed using the Chimera Uquime software. Suitable sequences were clustered into operational taxonomic units (OTUs) defined by a 3% distance level, based on the distance matrix. Taxonomic classification was performed with the SILVA 16S rRNA gene database (using the k-nearest neighbour consensus and Wang approaches). Confidence values of less than 80% (at a phylum level) were considered as unclassified according to Wang et al. (2007). The dataset containing the sequences was deposited in the BioSample database at the U.S. National Center for Biotechnology Information (NCBI), under the ID numbers SRR3645936 (Inoculum), SRR3646035 (CSTR-C), and SRR3646036 (CSTR-T).

DNA amplification from extracted DNA from rock samples was unsuccessful until date, even several protocols and modifications were tested. Failure could be due to the presence of copper ions adsorbed on the EPS matrix and released during the DNA extraction, something that potentially may damage the DNA. To evaluate the concentration of available copper in the sample, copper was extracted from 1 g of ground rock sample with 10 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA) at room temperature, in 50 ml polypropylene falcon tubes for 1 hr according to Lo and Yang (1999). Supernatants were filtered and quantified by Total Reflection X-Ray Fluorescence (TXRF), in the Interdepartmental Investigation Service (SIdI) at Universidad Autónoma de Madrid (Spain). Those analyses showed copper concentrations above 81.19 mg/l.

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Table S1. Detected prokaryotes in cultures from Las Cruces

Size	Phylum	Class	Order	Family	Genus	Total
Bacteria						
LC4-Mg1						
16368	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	unclassified	69.05
2298	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	9.69
1854	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfosporosinus	7.82
1115	Actinobacteria	Actinobacteria	Actinomycetales	Propionibactericeae	Propionibacterium	4.70
857	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	3.62
673	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfotomaculum	2.84
102	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	uncultured	0.43
76	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.32
44	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enteric Bacteria cluster	0.19
42	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfitobacterium	0.18
LC5-Mg1						
36009	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Thermincola	81.69
3972	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfotomaculum	9.01
1919	Actinobacteria	Actinobacteria	Actinomycetales	Propionibactericeae	Nocardioides	4.35
445	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	1.01
377	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.86
344	Actinobacteria	Actinobacteria	Actinomycetales	Propionibactericeae	Propionibacterium	0.78
173	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	unclassified	0.39
98	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Thermincola	0.22
95	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.22
85	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	unclassified	0.19
LC4-SBR1						
30268	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	99.54
52	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.17
7	Actinobacteria	Actinobacteria	Actinomycetales	Propionibactericeae	Propionibacterium	0.02
5	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.02
3	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	0.01
3	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.01
3	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.01
2	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.01

2	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	0.01
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	0.01
LC5-SBR1						
30319	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	91.17
1630	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix	4.90
52	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfosporosinus	0.16
28	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	0.08
22	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	0.07
16	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Incertae Sedis	0.05
9	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	0.03
6	Actinobacteria	Actinobacteria	Actinomycetales	Propionibactericeae	unclassified	0.02
5	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	0.02
5	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	0.02
Archaea						
LC4-Mg1						
11869	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	57.83
4275	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	20.83
1753	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	8.54
667	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	3.25
463	Euryarchaeota	Methanomicrobia	Methanomicrobiales	unclassified	unclassified	2.26
198	unclassified	unclassified	unclassified	unclassified	unclassified	0.96
141	unclassified	unclassified	unclassified	unclassified	unclassified	0.69
114	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	0.56
104	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	0.51
89	unclassified	unclassified	unclassified	unclassified	unclassified	0.43
LC5-Mg1						
11275	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	49.14
8742	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	38.10
1682	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	7.33
386	Euryarchaeota	Halobacteria	Halobacteriales	Halobacteriaceae	unclassified	1.68
86	unclassified	unclassified	unclassified	unclassified	unclassified	0.37
80	unclassified	unclassified	unclassified	unclassified	unclassified	0.35
58	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	0.25
58	unclassified	unclassified	unclassified	unclassified	unclassified	0.25

48	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	0.21
24	Euryarchaeota	unclassified	unclassified	unclassified	unclassified	0.10
LC5-SBR1						
10480	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	61.55
3237	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	19.01
1791	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	10.52
942	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	5.53
462	Euryarchaeota	Halobacteria	Halobacteriales	Halobacteriaceae	uncultured	2.71
9	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	0.05
5	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	0.03
5	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	0.03
4	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	0.02
4	Crenarchaeota	Soil Crenarchaeotic Group	unclassified	unclassified	unclassified	0.02