Supporting Online Material for

Manganese- and Iron-Dependent Marine Methane Oxidation

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Published 10 July 2009, *Science* **325**, 184 (2009)
DOI: 10.1126/science.1169984

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Materials and Methods

Sampling

Sediment samples were taken from two cruises to the Eel River Basin, one from July to August 2005 (R/V *Western Flyer*) and the second in September 2006 (R/V *Atlantis*). The samples used in the manganese experiments are from the first cruise: dive T-866 PC-42 (mat, 0-10 cm) and T-866 PC-73 (mat, 0-10 cm). The samples used in the iron experiments are from the second cruise: Dive A4254 PC 11 (mat, 0-6 cm) and PC-100 (*Calyptogena* clam, 0-12 cm). Sediment was sealed in mylar bags with argon on the ship and was refrigerated until use. Sediment used in the nitrate and ferric oxyhydroxide experiments was stored at 10º C for 6 months in sealed anaerobic mylar bags before incubation. Sediment for the birnessite and ferrihydrite experiments was stored at 10º C for 12 months in sealed anaerobic mylar bags before incubation.

Metal Synthesis

Ferric oxyhydroxide was synthesized by neutralizing a 0.4 M solution of $FeCl_3$ with sodium hydroxide (S*1*). Birnessite synthesis followed the protocol of Golden et al. (S*2*) 2-line Ferrihydrite was synthesized using the protocol in Cornell and Schwertmann (S*3*). Birnessite and ferrihydrite were sterilized using ethanol and were never autoclaved.

Incubations

Artificial sulfate-free seawater was made following the recipe of Lyman and Fleming (S*4*). Seawater was degassed with N_2/CO_2 and reduced with 0.5 g/L $Na_2S{\cdot}9H_20$. $FeCl_2$ was added in 1:1 stoichiometic ratio to the sulfide to precipitate all of the added sulfide and then the artificial seawater was autoclaved. The FeS was allowed to settle before use of the seawater so as to minimize the amount of sulfide added to the incubations. 10 mL of homogenized sediment was distributed to 120 mL culture bottles containing 30 mL of artificial seawater. Triplicate incubations were given sulfate (5 mM), birnessite (10 mM), ferrihydrite (10 mM), nitrate (5 mM), ferric oxyhydroxide (10 mM), or no provided electron acceptor. The headspace of the bottles was then filled with 2.5 bars methane. 35 mL of CO_2 and 6 mL ^{13}C-CH_4 was injected into the headspace. Isotope analysis of the CO_2 followed the same procedure used for similar studies (S*5*, S*6*). After ~7 months of incubation for the birnessite experiments, the headspace was vacuumed and a new headspace (with the same proportions of CO_2, ^{13}C-CH_4, and CH_4) was added to all of these bottles. The data in the figures starts at the time the new headspace was added and are normalized to the $^{13}F_{CO2}$ values at this time. This makes for a total of 10 months that each bottle in the birnessite experiments was incubated. The ferrihydrite experiments also were given a new headspace (as described with the manganese experiments) after 5 months. The data shown the in the ferrihydrite figures begins at the time when the new headspace was added and the data is normalized to this starting time point. We changed the headspace in both the birnessite and ferrihydrite experiments, and thus created a new beginning to the experiments, after months of incubation to ensure that any residual sulfate in the mud had been consumed at the start of our experiments. Additional sulfate was injected into the sulfate incubations, and birnessite and ferrihydrite into their respective incubations when the headspace was changed.

Free Energy Calculations

The *in situ* conditions used in the free energy calculation for the manganese experiments are: $[MnO_2]$ = 10 mM, $[Mn^{2+}]$ = 0.2 mM, $[H^+] = 10^{-8}$ mM, $[HCO_3^-]$ =11 mM, $[CH_4]$ = 1.5 mM. The *in situ* conditions used in the free energy calculation for the iron experiments are: $[Fe(OH)_3]$ = 10 mM, $[Fe^{2+}]$ = 10 mM, $[H^+] = 10^{-8}$ mM, $[HCO_3^-]$ =11 mM, $[CH_4]$ = 1.5 mM. The *in situ* conditions used in the free energy calculations for the sulfate incubations are: $[SO_4^{2-}]$ = 5 mM, $[HS^-]$ =2 mM, $[H^+] = 10^{-8}$ mM, $[HCO_3^-]$ =11 mM, and $[CH_4]$ = 1.5 mM. Concentration of MnO_2, SO_4^{2-}, $Fe(OH)_3$, HS^- are based on the amount of each of these species added at the start of the incubations. The values for Mn^{2+} and Fe^{2+} are based averaged measured values of starting concentrations of these species. Values of HCO_3^- and CH_4 were calculated using Henry's law. The value for H^+ concentration comes from pH measurements of the incubations. We estimate that each of the incubations contain 7 cm^3 of sediment. The temperature in the free energy calculations was 10º C for both experiments.

Dissolved manganese and iron measurements

1 mL of sample from the incubation was taken for dissolved manganese measurements. This sample was immediately acidified to pH 2 using HCL, and filtered on a 2 μm filter. Dissolved manganese was then measured on a Leeman Labs PS3000UV ICP (inductively coupled plasma emission spectrometer). 0.5 mL samples were taken for dissolved iron measurements and acidified to pH 2 using HCL and then centrifuged for 5 minutes at 14,000 rpm to remove sediment. Dissolved iron was measured using the ferrozine assay (S*7*).

Clone libraries/Phylogenetic Analysis

Samples were taken from the starting sediment used in all incubations as well as at the end of the experiments (which corresponds to a total of ~10 months of incubation). Bacterial primers 27F 5-'AGAGTTTGATCCTGGC-3' and 1492R 5'-GGTTACCTTGTTACG-3', archaeal primers 21F 5'-TTCCGGTTGATCCYGCCGGA-3' and 958R 5'-YCCGGCGTTGAMTCCAATT-3' *mcr*A primer sequences are *mcr*A forward 5'-GGTGGTGTMGGATTCACACAR-3' and *mcr*A reverse 5'-TCATTGCRTAGTTWGGRTAGTT-3' (S*8*) were used in PCR amplification of DNA using PuRe Taq Ready-to-Go PCR beads (GE Healthcare). Amplification conditions for archaeal PCR are initial melting at 94º for 5 min, followed by 30 cycles of 94º for 1 min, 58º for 1 min, and 72º for 1.74 min, ending in a final elongation at 72º for 20 min, bacterial PCR conditions are described in (S*9*) and for *mcr*A in Moran et al. (S*6*). DNA extraction, cloning and sequencing was performed as described by Moran et al. (S*5*). Archaea were classified based on the classification presented in Knittel et al. (2004) (S*10*). Possible metabolisms for bacteria were based on phylogenetic association of clones determined using the greengenes classify tool (http://greengenes.lbl.gov) as well as BLAST hits using the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi. Bacterial neighbor joining trees were constructed using PAUP. Closest sequences to our *mcr*A sequences were found using BLAST and identified using *mcr*A sequences that had already been classified (S*11*). We screened 46 archaeal, 47 bacterial, 46 *mcr*A and clones from the original sediment, 24 archaeal, 23 bacteria and 24 *mcr*A clones from the live control, 31 archaeal, 31 bacterial, and 32 *mcr*A clones from the manganese incubations, and 23 archaeal, 24 bacterial, and 24 *mcr*A clones from the sulfate incubations.

Text

Additional manganese and iron sources to the Eel River Basin (ERB)

Aeolian dust deposition to ocean basins is estimated at 1000 – 2000 Tg/yr (S*12*). If dust composition reflects the composition of the Earth, it can be estimated that 50 - 100 Tg/yr of iron and 1 - 2 Tg/yr manganese are deposited to oceans. This is a significant amount of both iron and manganese, but it is about of an order of magnitude less than the riverine input. The Gorda Ridge has the potential to deliver an additional source of both iron and manganese to the ERB. It is estimated that hydrothermal sources release 0.6 – 1.9 Tg dissolved manganese and 1.3 -11 Tg dissolved iron into oceans (S*13*), which, once oxidized, has the oxidative potential to consume 0.03 % – 0.7 % of methane globally consumed by AOM today. Although this amount of oxidative potential seems very small, especially compared to riverine input, iron and manganese can be oxidized and reduced 100 – 300 times before being buried (S*14*). Thus, the hydrothermal flux of iron and manganese into the oceans has the ability to oxidize significantly more methane than a direct stoichiometric relationship would imply.

Presence of dissolved manganese

The incubations with birnessite show an increase in the amount of dissolved manganese with time. They also have a greater amount of dissolved manganese than the autoclaved control, which shows no change in dissolved manganese with time. The live controls, with no electron acceptor addition, show no measurable dissolved manganese. The concentration of dissolved mangnaese only increases by approximately 5 μM, which is ⅒ of what one would expect to see based on stoichiometric calculations. However, one expects that most of the dissolved manganese in our incubations will precipitate as manganese carbonates (such as rhodochrosite) (S*15*). If the precipitation of manganese carbonates is taken into account, the lower concentration of dissolved manganese observed in the incubations is not surprising. Similarly, it is expected that iron carbonates (such as siderite) (S*15*) will precipitate in our incubations as ferrihydrite is reduced. Thus, we would also expect to see a small change in the amount of dissolved iron in our cultures. The starting concentration of dissolved iron was 10 mM and thus the expected change of the dissolved iron concentration (on the order of a few μM) is only a few tenths of a percent change. It is likely for this reason that we did not observe a significant increase in dissolved iron in the ferrihydrite incubations.

Supporting Figures and Legends

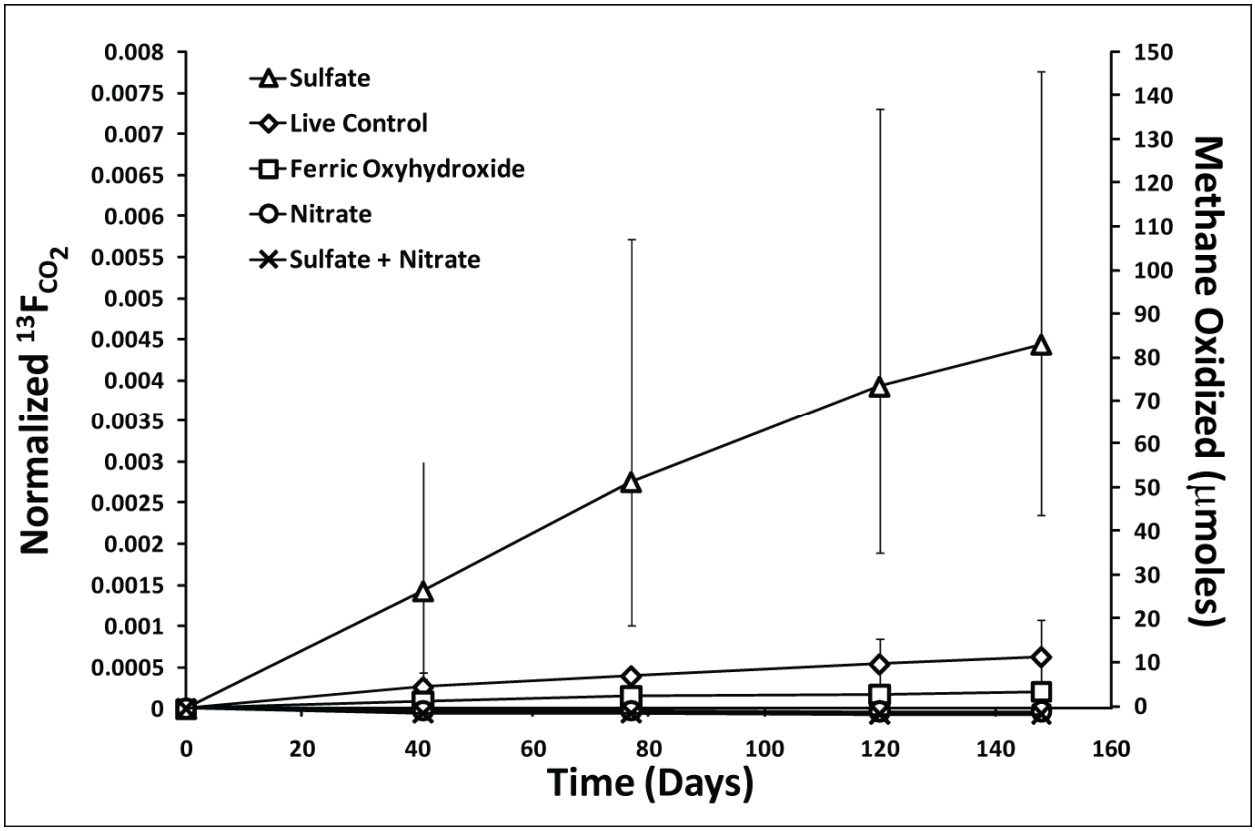


Figure S1. ^{13}C enrichment of CO_2 reported in $^{13}F_{CO2}$ ($^{13}C/^{13}C+^{12}C$) values and converted to moles methane oxidized. The incubations with ferric oxyhydroxide, nitrate, and nitrate with sulfate oxidize less methane than the live control (sulfate free, no provided electron acceptor), indicating that ferric oxyhydroxide and nitrate cannot be used as an electron acceptor in AOM. In addition, because there is no methane oxidation with nitrate and sulfate, these data indicate that nitrate inhibits AOM. Sediment was stored at 10^{o} C for 12 months in sealed anaerobic mylar bags before incubation. Error bars represent the range of data from the triplicate incubations.

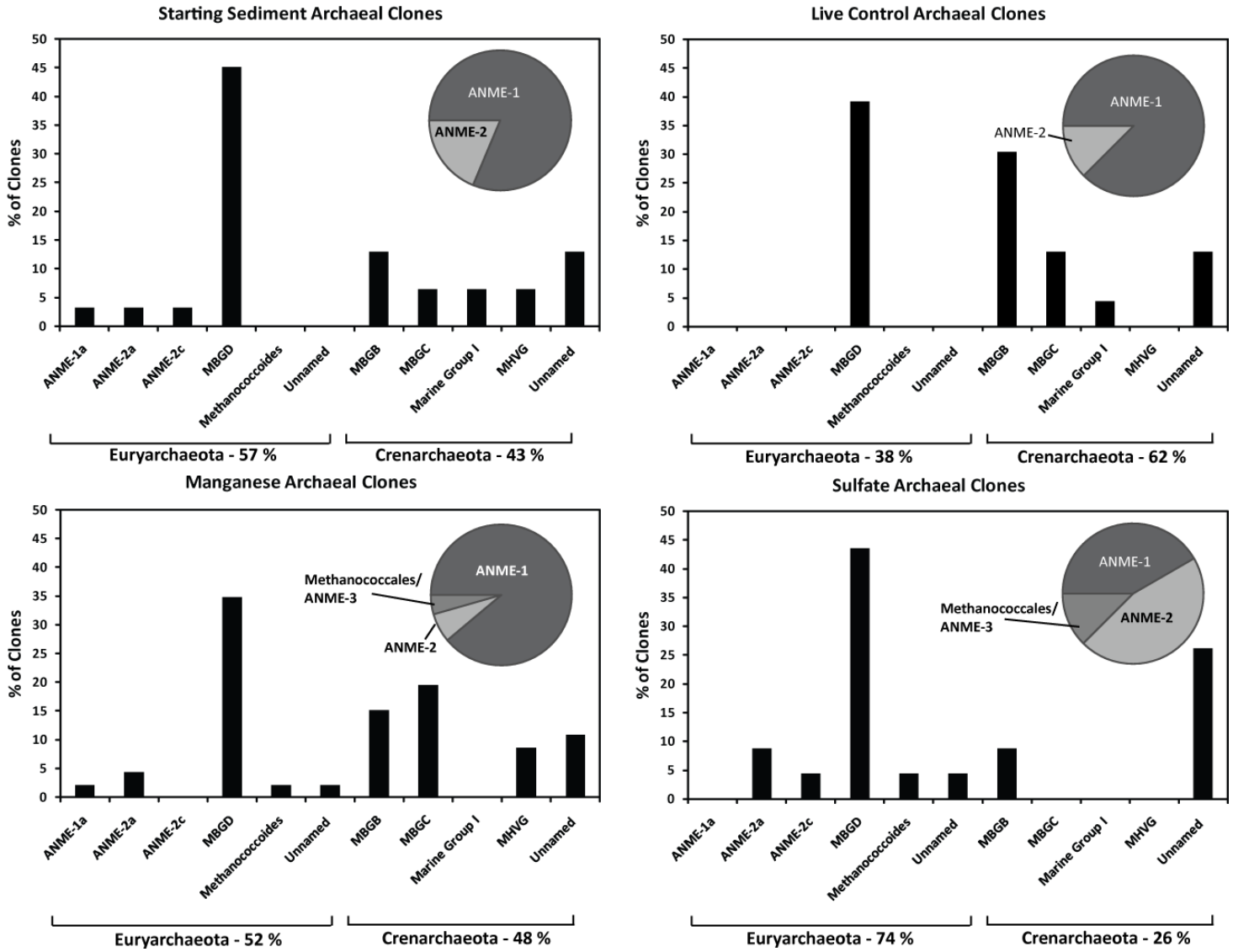
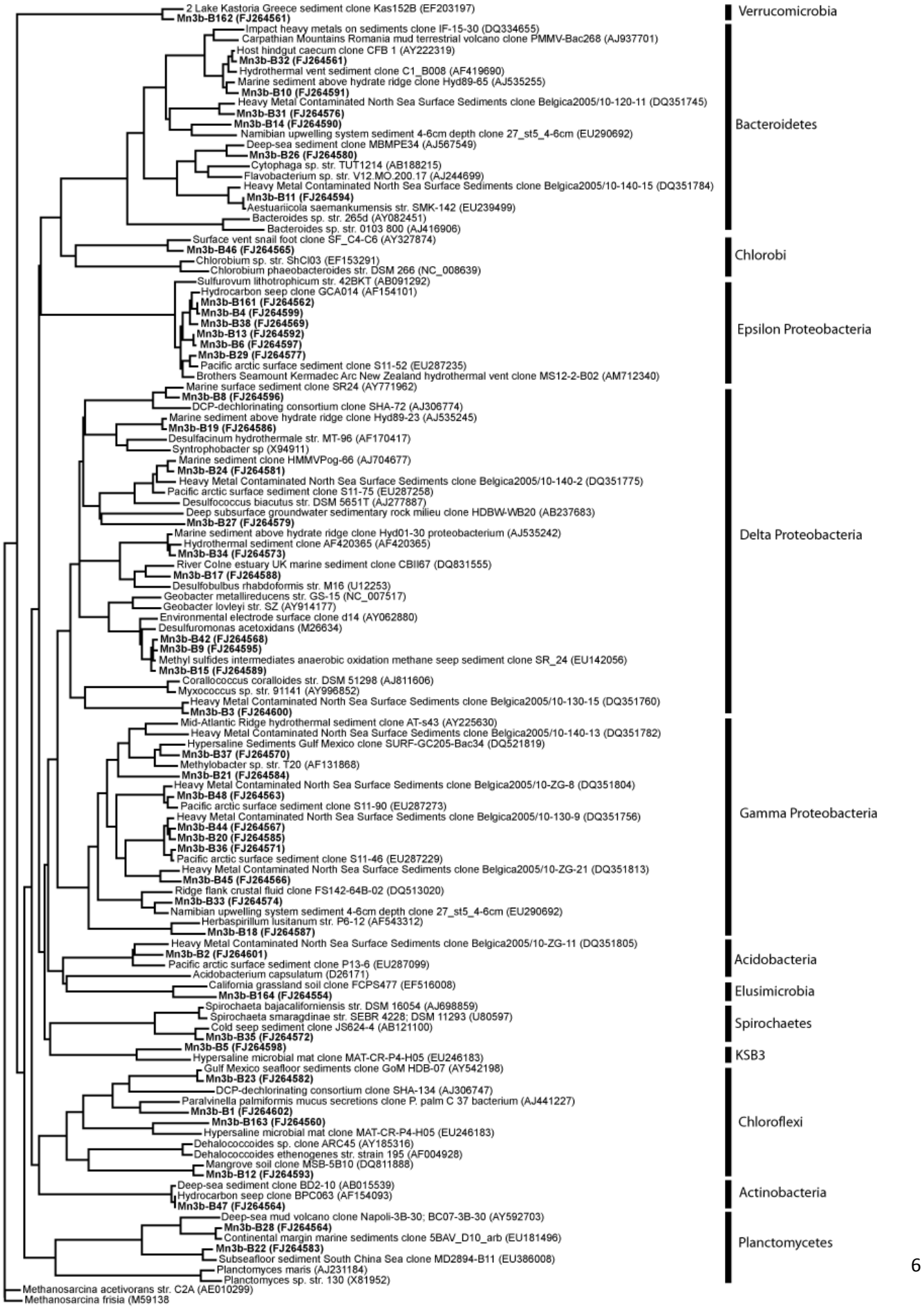


Figure S2. Percentages of archaeal clones from the initial sediment, live control, manganese, and sulfate incubations, based on 16S rRNA and the mcrA gene (insert). MBGD=Marine Benthic Group D, MBGB=Marine Benthic Group B, MBGC=Marine Benthic Group C. The starting sediment was stored anaerobically for approximately a year before it was used, and therefore this microbial characterization does not accurately reflect the proportions of archaea when it was sampled from the Eel River Basin.

Figure S3. (Shown on pg. 6) Neighbor joining phylogentic tree of bacterial clones associated with the manganese incubations (Mn3b-B clones). Phylogenetic analysis included the top BLAST matches and other nearest neighbors (with emphasis on metal-associated sequences).


2 Lake Kastoria Greece sediment clone Kas152B (EF203197)
Mn3b-B162 (FJ264561)
Verrucomicrobia
Impact heavy metals on sediments clone IF-15-30 (DQ334655)
Carpathian Mountains Romania mud terrestrial volcano clone PMMV-Bac268 (AJ937701)
Host hindgut caecum clone CFB 1 (AY222319)
Mn3b-B32 (FJ264561)
Hydrothermal vent sediment clone C1_B008 (AF419690)
Marine sediment above hydrate ridge clone Hyd89-65 (AJ535255)
Mn3b-B10 (FJ264591)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-120-11 (DQ351745)
Mn3b-B31 (FJ264576)
Mn3b-B14 (FJ264590)
Namibian upwelling system sediment 4-6cm depth clone 27_st5_4-6cm (EU290692)
Deep-sea sediment clone MBMPE34 (AJ567549)
Mn3b-B26 (FJ264580)
Cytophaga sp. str. TUT1214 (AB188215)
Flavobacterium sp. str. V12.MO.200.17 (AJ244699)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-140-15 (DQ351784)
Mn3b-B11 (FJ264594)
Aestuariicola saemankumensis str. SMK-142 (EU239499)
Bacteroides sp. str. 265d (AY082451)
Bacteroides sp. str. 0103 800 (AJ416906)
Bacteroidetes
Surface vent snail foot clone SF_C4-C6 (AY327874)
Mn3b-B46 (FJ264565)
Chlorobium sp. str. ShCl03 (EF153291)
Chlorobium phaeobacteroides str. DSM 266 (NC_008639)
Chlorobi
Sulfurovum lithotrophicum str. 42BKT (AB091292)
Hydrocarbon seep clone GCA014 (AF154101)
Mn3b-B161 (FJ264562)
Mn3b-B4 (FJ264599)
Mn3b-B38 (FJ264569)
Mn3b-B13 (FJ264592)
Mn3b-B6 (FJ264597)
Mn3b-B29 (FJ264577)
Pacific arctic surface sediment clone S11-52 (EU287235)
Brothers Seamount Kermadec Arc New Zealand hydrothermal vent clone MS12-2-B02 (AM712340)
Epsilon Proteobacteria
Marine surface sediment clone SR24 (AY771962)
Mn3b-B8 (FJ264596)
DCP-dechlorinating consortium clone SHA-72 (AJ306774)
Marine sediment above hydrate ridge clone Hyd89-23 (AJ535245)
Mn3b-B19 (FJ264586)
Desulfacinum hydrothermale str. MT-96 (AF170417)
Syntrophobacter sp (X94911)
Marine sediment clone HMMVPog-66 (AJ704677)
Mn3b-B24 (FJ264581)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-140-2 (DQ351775)
Pacific arctic surface sediment clone S11-75 (EU287258)
Desulfococcus biacutus str. DSM 5651T (AJ277887)
Deep subsurface groundwater sedimentary rock milieu clone HDBW-WB20 (AB237683)
Mn3b-B27 (FJ264579)
Marine sediment above hydrate ridge clone Hyd01-30 proteobacterium (AJ535242)
Hydrothermal sediment clone AF420365 (AF420365)
Mn3b-B34 (FJ264573)
River Colne estuary UK marine sediment clone CBII67 (DQ831555)
Mn3b-B17 (FJ264588)
Desulfobulbus rhabdoformis str. M16 (U12253)
Geobacter metallireducens str. GS-15 (NC_007517)
Geobacter lovleyi str. SZ (AY914177)
Environmental electrode surface clone d14 (AY062880)
Desulfuromonas acetoxidans (M26634)
Mn3b-B42 (FJ264568)
Mn3b-B9 (FJ264595)
Methyl sulfides intermediates anaerobic oxidation methane seep sediment clone SR_24 (EU142056)
Mn3b-B15 (FJ264589)
Corallococcus coralloides str. DSM 51298 (AJ811606)
Myxococcus sp. str. 91141 (AY996852)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-130-15 (DQ351760)
Mn3b-B3 (FJ264600)
Delta Proteobacteria
Mid-Atlantic Ridge hydrothermal sediment clone AT-s43 (AY225630)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-140-13 (DQ351782)
Hypersaline Sediments Gulf Mexico clone SURF-GC205-Bac34 (DQ521819)
Mn3b-B37 (FJ264570)
Methylobacter sp. str. T20 (AF131868)
Mn3b-B21 (FJ264584)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-ZG-8 (DQ351804)
Mn3b-B48 (FJ264563)
Pacific arctic surface sediment clone S11-90 (EU287273)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-130-9 (DQ351756)
Mn3b-B44 (FJ264567)
Mn3b-B20 (FJ264585)
Mn3b-B36 (FJ264571)
Pacific arctic surface sediment clone S11-46 (EU287229)
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-ZG-21 (DQ351813)
Mn3b-B45 (FJ264566)
Ridge flank crustal fluid clone FS142-64B-02 (DQ513020)
Mn3b-B33 (FJ264574)
Namibian upwelling system sediment 4-6cm depth clone 27_st5_4-6cm (EU290692)
Herbaspirillum lusitanum str. P6-12 (AF543312)
Mn3b-B18 (FJ264587)
Gamma Proteobacteria
Heavy Metal Contaminated North Sea Surface Sediments clone Belgica2005/10-ZG-11 (DQ351805)
Mn3b-B2 (FJ264601)
Pacific arctic surface sediment clone P13-6 (EU287099)
Acidobacterium capsulatum (D26171)
Acidobacteria
California grassland soil clone FCPS477 (EF516008)
Mn3b-B164 (FJ264554)
Elusimicrobia
Spirochaeta bajacaliforniensis str. DSM 16054 (AJ698859)
Spirochaeta smaragdinae str. SEBR 4228; DSM 11293 (U80597)
Cold seep sediment clone JS624-4 (AB121100)
Mn3b-B35 (FJ264572)
Spirochaetes
Mn3b-B5 (FJ264598)
Hypersaline microbial mat clone MAT-CR-P4-H05 (EU246183)
KSB3
Gulf Mexico seafloor sediments clone GoM HDB-07 (AY542198)
Mn3b-B23 (FJ264582)
DCP-dechlorinating consortium clone SHA-134 (AJ306747)
Paralvinella palmiformis mucus secretions clone P. palm C 37 bacterium (AJ441227)
Mn3b-B1 (FJ264602)
Mn3b-B163 (FJ264560)
Hypersaline microbial mat clone MAT-CR-P4-H05 (EU246183)
Dehalococcoides sp. clone ARC45 (AY185316)
Dehalococcoides ethenogenes str. strain 195 (AF004928)
Mangrove soil clone MSB-5B10 (DQ811888)
Mn3b-B12 (FJ264593)
Chloroflexi
Deep-sea sediment clone BD2-10 (AB015539)
Hydrocarbon seep clone BPC063 (AF154093)
Mn3b-B47 (FJ264564)
Actinobacteria
Deep-sea mud volcano clone Napoli-3B-30; BC07-3B-30 (AY592703)
Mn3b-B28 (FJ264564)
Continental margin marine sediments clone 5BAV_D10_arb (EU181496)
Mn3b-B22 (FJ264583)
Subseafloor sediment South China Sea clone MD2894-B11 (EU386008)
Planctomyces maris (AJ231184)
Planctomyces sp. str. 130 (X81952)
Planctomycetes
Methanosarcina acetivorans str. C2A (AE010299)
Methanosarcina frisia (M59138
0.1

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