Running title: Biological sedimentary iron oxides

Subject: Geomicrobiology and microbial contributions to geochemical cycles

A biological source of marine sedimentary iron oxides

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Abstract

The biogeochemical cycle of iron is intricately linked to numerous element cycles. Although reductive biological processes that bridge the iron cycle to other element cycles are established, little is known about microbial oxidative processes on iron cycling in sedimentary environments—resulting in the formation of iron oxides. Here, we show that a major source of sedimentary iron oxides originates from the metabolic activity of iron-oxidizing bacteria from the class Zetaproteobacteria, stimulated by burrowing animals in coastal sediments. Zetaproteobacteria were estimated to be a global total of 10^{26} cells in coastal, bioturbated sediments and would equate to an annual production of approximately 7.9 x 10^{15} grams of sedimentary iron oxides—twenty-five times larger than the annual flux of iron oxides by rivers. These data suggest that iron-oxidizing Zetaproteobacteria are keystone organisms in marine sedimentary environments given their low numerical abundance; yet exert a profound impact via the production of iron oxides.

Keywords: iron-oxidizing bacteria/Zetaproteobacteria/bioturbation/iron biogeochemistry/keystone organism

Main

Iron oxides are important components of coastal and continental shelf sediments, and are thought to originate primarily by river deposition (Poulton and Raiswell, 2002). Authigenic iron oxides can also be formed by the oxidation of ferrous iron [Fe(II)], which has been largely attributed to chemical oxidation of Fe(II) in pore waters in areas with significant sediment mixing and irrigation by animals—bioturbation and bioirrigation—and subsequent reaction with oxygen in anoxic sediments (Aller, 1982; Canfield, 1989). Although sedimentary chemical oxidation of iron is important under saturated oxygen conditions at neutral pH (Canfield, 1989), bioirrigated sediments contain microenvironments—formed by burrowing animals—that are well below saturation (Kristensen and Kostka, 2005) where the biological contribution to iron oxidation is quantitatively more significant (Emerson *et al.*, 2010). Under low-oxygen conditions (< 100 μM O_2) and without rapid replenishment of highly reactive, poorly-crystalline iron oxides (i.e., ferrihydrite and lepidocrocite), they would be quickly exhausted by hydrogen sulfide and by bacterial iron reduction, and form iron sulfides or be released to the water column. Collectively, these findings suggest that biology is involved and important in marine sedimentary iron oxidation under ferruginous conditions commonly observed in bioturbated sediments.

The Zetaproteobacteria represent a class of iron-oxidizing bacteria (FeOB) that are exclusively found in marine or saline-influenced environments that contain high ferrous iron [Fe(II)] concentrations (McAllister *et al.*, 2011; McBeth *et al.*, 2013; Scott *et al.*, 2015, 2017). Coastal marine sediments can have Fe(II) pore water concentrations ranging from ~1-2,000 μmol L^{-1} that are capable of supporting lithoautotrophic populations of Zetaproteobacteria (Emerson, 2016). Recent studies have identified Zetaproteobacteria in surface openings of benthic macrofauna in the Mediterranean Sea (Rubin-Blum *et al.*, 2014), worm burrows in sub-marine

67 groundwater discharge into sands in Delaware (McAllister *et al.*, 2015), coastal sediments in
68 Denmark (Laufer *et al.*, 2016), and Baltic and North Sea sediments (Reyes *et al.*, 2016). These
69 recent studies suggest that Zetaproteobacteria may play a significant role in iron oxidation in
70 marine sediments—a quantitative estimate of their abundance is necessary to determine their
71 biogeochemical role on a global scale.

72 We analyzed the coastal sediment microbial communities to determine the extent of
73 Zetaproteobacteria from geographically diverse sites (n=90; Supplementary Table 1) utilizing
74 16S rRNA gene sequencing, which highlights their importance on a global scale. Iron oxidation
75 appears ubiquitous in the Zetaproteobacteria (Field *et al.*, 2015; Scott *et al.*, 2015; Barco *et al.*,
76 2015), thus the 16S rRNA gene can be used to infer this specific metabolism. We also enriched
77 for environmentally relevant iron-oxidizing bacteria from coastal sediments, which provided
78 further metabolic evidence of the importance of iron oxidation in marine sediments. A meta-
79 analysis of 16S rRNA gene studies revealed the extent and importance of Zetaproteobacteria on
80 the global sedimentary iron biogeochemical cycle.

81 We identified Zetaproteobacteria in 60 % of our samples (Supplementary Table S1), and
82 their median relative abundance was 1.1 percent of the total microbial community (range = 0.04-
83 15 %) in worm (e.g., polychaetes) burrows in coastal marine sediments (Fig. 1a).
84 Zetaproteobacteria were ten times less abundant in bulk sediments (Fig. 1a) with a median near
85 zero percent (range = 0-1 %), and were statistically different from worm burrows (p-value=9.2 x
86 10^{-7}, Wilcoxon test). The large ranges (0.04-15 %) and non-normal distribution of
87 Zetaproteobacteria relative abundance in worm burrows (see Supplementary Materials and
88 Methods) was most likely a combination of differences in burrow ventilation rates and
89 efficiencies (Kristensen and Kostka, 2005), differences in sediment physicochemical conditions

(Supplementary Table S1), and sampling bias (for example, residual sediment on worm burrows). Bioirrigation by benthic animals increases the extent of oxidative processes in these sediments, thus biological iron oxidation can occur at greater depths (10s of centimeters) than typical oxygen penetration of a few millimeters into coastal surface sediments. The abundance of Zetaproteobacteria at the burrow walls correlated with the concentration of pore water ferrous iron [Fe(II)] (Fig. 1b), which is their main energy source—resulting in the production of solid phase iron oxides around worm burrows (Fig. 1c). Quantitatively, highly reactive, iron oxides—operationally extractable by sodium dithionite (Poulton and Canfield, 2005)—were 3 times higher at burrow walls, which accounted for 20-40 % of the iron oxides with depth (Supplementary Figure S1 and S2). These freshly-produced iron oxides are important substrates for iron-reducing microorganisms that release Fe(II) into pore waters, and are essential to the supply of dissolved iron (dFe) to phytoplankton in the water column in coastal communities and continental shelves (Severmann *et al.*, 2010). Iron oxides are also important to the mineralization of organic matter in marine sediments by iron reducers (Canfield, 1989) substrates for early pyrite diagenesis (Berner, 1984), enhance organic matter burial (Lalonde *et al.*, 2012), and inhibit accumulation of pore water hydrogen sulfide, preventing conditions detrimental to benthic animals (Kristensen and Kostka, 2005), perhaps functioning as a local firewall against euxinic conditions(Seitaj *et al.*, 2015).

The relative abundance of Zetaproteobacteria in worm burrows resembles the Fe(II) concentration profile (Supplementary Figure S3), and both were at their maximum values around 2-3 cm. The high Fe(II) (~40-140 μM) and low oxygen (~20-60 μM) conditions present in bioturbated sediment pore waters (Supplementary Figure S3) were ideal habitats for microaerophilic Zetaproteobacteria to thrive (Emerson *et al.*, 2010). Zetaproteobacteria relative

abundance decreased with depth in both burrows and sediments (Supplementary Fig. S3), likely due to the decrease in Fe(II) with depth and increase in hydrogen sulfide production by sulfate-reducing bacteria. Although there is oxygen in these sediments at depth, hydrogen sulfide may inhibit oxygen respiratory machinery under these conditions. The formation of iron sulfide minerals with increasing depth by biogenic sulfide may also compete with Zetaproteobacteria for access to Fe(II). Under these ferruginous settings, biotic rates of Fe(II) oxidation exceed abiotic chemical oxidation (Emerson *et al.*, 2010).

Two Zetaproteobacteria Operational Taxonomic Units (herein, referred to as ZetaOTUs) dominated the Zetaproteobacterial diversity in worm burrows (Supplementary Table S2). The dominant ZetaOTU across all samples was ZetaOTU14, which comprised 32 % of all ZetaOTUs (Supplementary Table S2), and is represented by four single cell amplified genomes (SAGs) from diffuse flow vent systems (Field *et al.*, 2015; Scott *et al.*, 2015, 2017). We isolated the first member of ZetaOTU14, strain CSS-1 from iron oxide surface flocculent in a laboratory bioturbation microcosm (Supplementary Figure S4). This strain grew best under low oxygen (~60 μM O_2) and high Fe(II) concentrations similar to those measured from sediment pore waters (Supplementary Figure S3). Strain CSS-1 produced stalks encrusted with poorly-crystalline iron oxides under laboratory conditions (Supplementary Figure S4). These iron oxides are consistent with those produced by other Zetaproteobacteria (Chan *et al.*, 2010), as well as in naturally occurring iron mats associated with hydrothermal vents, which are highly reactive, and resistant to undergoing diagenesis to more crystalline oxides (e.g., goethite) (Picard *et al.*, 2015). Single cell genomes from ZetaOTU14 representatives contained genes essential for growth on iron and low oxygen conditions (Supplementary Table S2). The second most abundant OTU was ZetaOTU9 (22 %) and is represented by two cultured isolates (*Ghiorsea bivora* strains TAG-1

136 and SV108) (Mori *et al*.,, 2017), as well as 5 SAGs from deep-sea vents (Supplementary Table
137 S2). ZetaOTU9 isolates also had genes necessary for growth on iron and low oxygen
138 (Supplementary Table S2), and have also been shown to oxidize hydrogen, which may explain
139 the ubiquity of this OTU in sediments and other environments (see below). There was no clear
140 distribution of ZetaOTUs 14 and 9 with respect to depth (Supplementary Figure S3) in worm
141 burrows and sediments—although it is likely that they are adapted for specific Fe(II) and O_2
142 concentrations, which were hypothesized for other Zetaproteobacteria (Field *et al.*, 2015).
143 We searched for Zetaproteobacterial 16S rRNA gene sequences in marine sediment
144 datasets (Supplementary Table S3), and identified them in numerous sediments on a global scale
145 (Figure 2). We found a pattern consistent with our samples—ZetaOTUs 14 and 9 were present
146 and generally the most abundant ZetaOTUs in coastal and shelf sediments (Figure 2).
147 Zetaproteobacteria relative abundance was not found to exceed one percent in other studies, as
148 microenvironments were not considered, which are abundant in bioturbated sediments
149 (Kristensen and Kostka, 2005). Accordingly, we hypothesize that when the abundance of
150 Zetaproteobacteria exceeds ~0.1 % in sediments, there is active growth and iron oxidation
151 associated with bioturbating and bioirrigating animals. We estimated a median global population
152 size of Zetaproteobacteria to be 1.05×10^{26} cells (range = 3.83×10^{24}-1.44×10^{27} cells) from our
153 measurements (Fig. 1a) and from other studies (Fig. 2) in continental shelf sediments using
154 cellular abundance in the upper 10 centimeters—the worldwide average depth of bioturbation
155 (Boudreau, 1998)—of continental shelf environments (total cells =10^{29}; <150 m water depth
156 (Kallmeyer *et al.*, 2012)). The global Zetaproteobacteria abundance estimate was then used with
157 recent iron oxide production rate measurements from diffuse flow vents (~1.3×10^{-16} mol Fe cell^{-1}
158 hr^{-1}) (Emerson *et al.*, 2016), which could result in the production of ~7 petagrams of iron

159 oxides per year (range = 0.1-70 petagrams per year). Recent two-dimensional, sub-millimeter
160 Fe(II) measurements in bioturbated sediments revealed extensive Fe(II) oxidation occurring
161 within the immediate vicinity of worm burrows and a rapid re-oxidation rate of 3.78 ±1.4 mmol
162 Fe m^{-2} day^{-1} (de Chanvalon *et al.*, 2017). These chemical rate measurements combined with an
163 estimate of the global volume of bioturbated coastal sediments 10 cm deep (~2.1 x 10^{13} m^3)
164 (Teal *et al.*, 2008) would equate to an annual production of 1.6 ± 1.1 petagrams of iron oxides.
165 These two independent estimations of iron oxide production rates are well within the range of
166 one another. Based on these estimates, the annual biological oxidation of iron in sediments—
167 forming iron oxides—could exceed the annual flux of iron oxides from rivers to coastal
168 sediments (Poulton and Raiswell, 2002) up to a factor of twenty-five.
169 Zetaproteobacteria exert a profound impact on global sedimentary biogeochemistry via
170 the production of biogenic, highly-reactive iron oxides despite their low global abundance (~0.11
171 %)—effectively functioning as keystone organisms in coastal sediments stimulated by burrowing
172 animals. Zetaproteobacteria contribute significantly to the rapid rates of Fe(II) re-oxidation
173 measured and observed in coastal sediments (Chanvalon *et al.*, 2017). Climate change outcomes
174 such as coastal hypoxia may have positive or negative effects on the sedimentary iron
175 biogeochemical cycle—either stimulating microaerobic bacterial iron oxidation resulting in an
176 increase in iron oxide production, thus enhancing dFe release or inhibiting oxidation by the
177 increase in hydrogen sulfide production, precipitating Fe as iron sulfides. The result of an
178 increase or decrease in dFe flux would be enhanced or reduced primary productivity by
179 phytoplankton, respectively. Thus, sedimentary iron oxide formation by Zetaproteobacteria may
180 have a direct impact on important water column processes such as carbon and nitrogen fixation.
181

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The authors declare no conflict of interest.

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Figure Legends

Figure 1. Boxplots of the relative abundance of Zetaproteobacteria (**a**) in iron-oxide lined worm burrow walls (n=29) and surrounding sediments (n=61). Notches are representative of 95 % confidence interval and the medians (solid black lines) between worm burrows and sediments (1.1 % and 0 %, respectively) are statistically different (p-value=9.2 x 10^{-7}, Wilcoxon test). Filled circles represent individual data points and open circles indicate outliers. Zetaproteobacteria relative abundance (%) as a function of pore water ferrous iron [Fe(II)] concentration (µmol L^{-1}) (**b**) from worm burrows (blue circles, fitted blue line, orange fill = 95 %

274 confidence interval) and sediments (black circles, fitted black line, grey fill = 95 % confidence
275 interval) (see Methods for details on line fits). Characteristic iron oxide lined worm burrow walls
276 (c) from "The Eddy", Sheepscot River, Maine, USA (image from 27 August 2015). Burrow
277 walls are likely created by the polychaete, *Nereis diversicolor* or hemichordate, *Saccoglossus*
278 *kowalevskii,* which are both common to these intertidal sediments in Maine.
279 **Figure 2.** Global distribution of Zetaproteobacteria in marine sediments (circles) and non-
280 sediment sites (triangles) such as hydrothermal vents. The relative abundance of
281 Zetaproteobacteria in sediments from other 16S rRNA gene studies was never above 1 % and
282 was typically within the range measured from bulk marine sediments from Maine. Sequences are
283 from numerous studies (Supplementary Table S3) that include Sanger, 454, and Illumina
284 sequencing technologies.

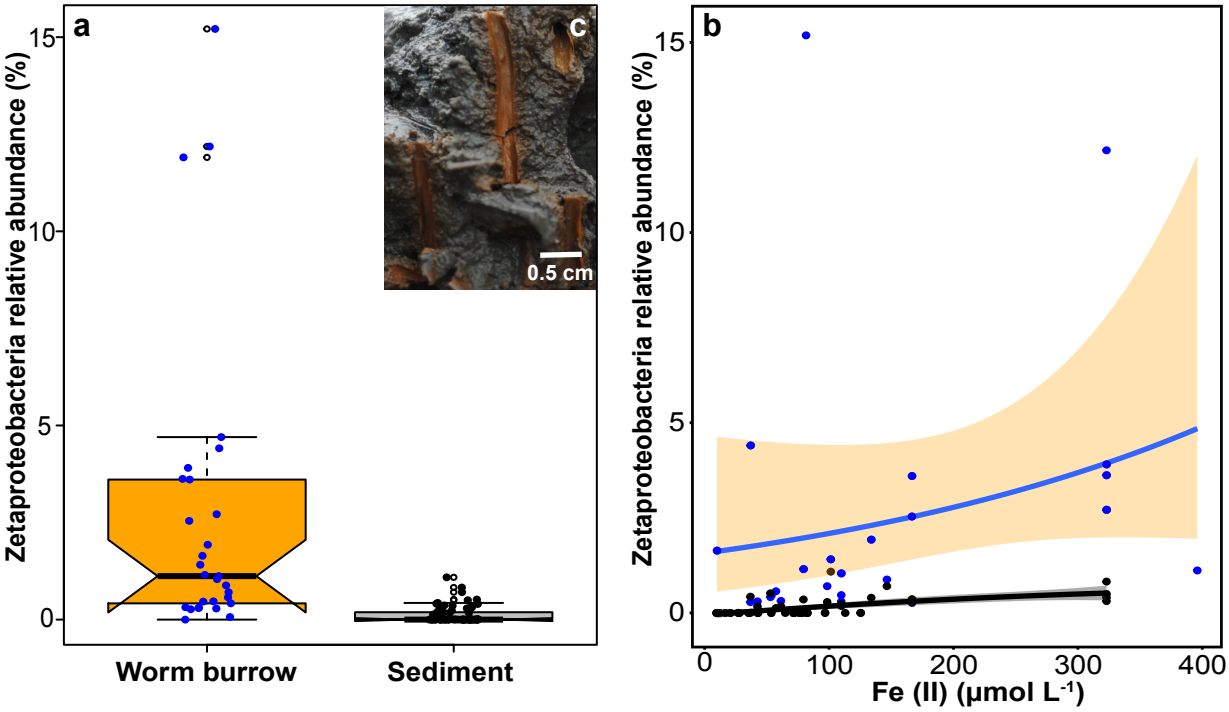

a
c
0.5 cm
Zetaproteobacteria relative abundance (%)
15
10
5
0
Worm burrow
Sediment
b
Zetaproteobacteria relative abundance (%)
15
10
5
0
0
100
200
300
400
Fe (II) (μmol L-1)

Figure 1

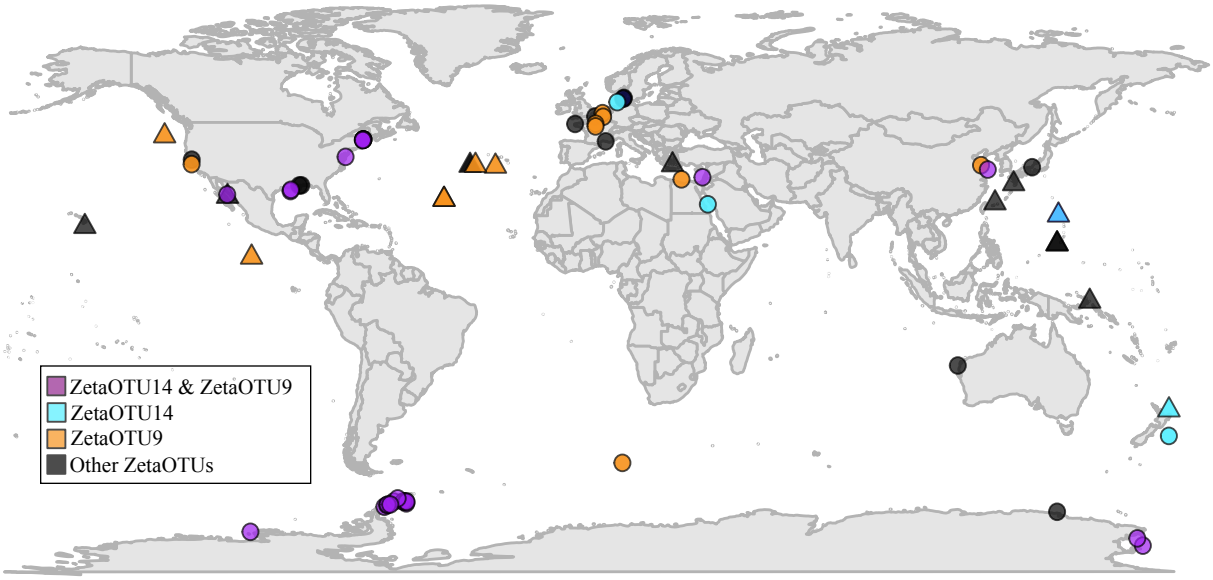
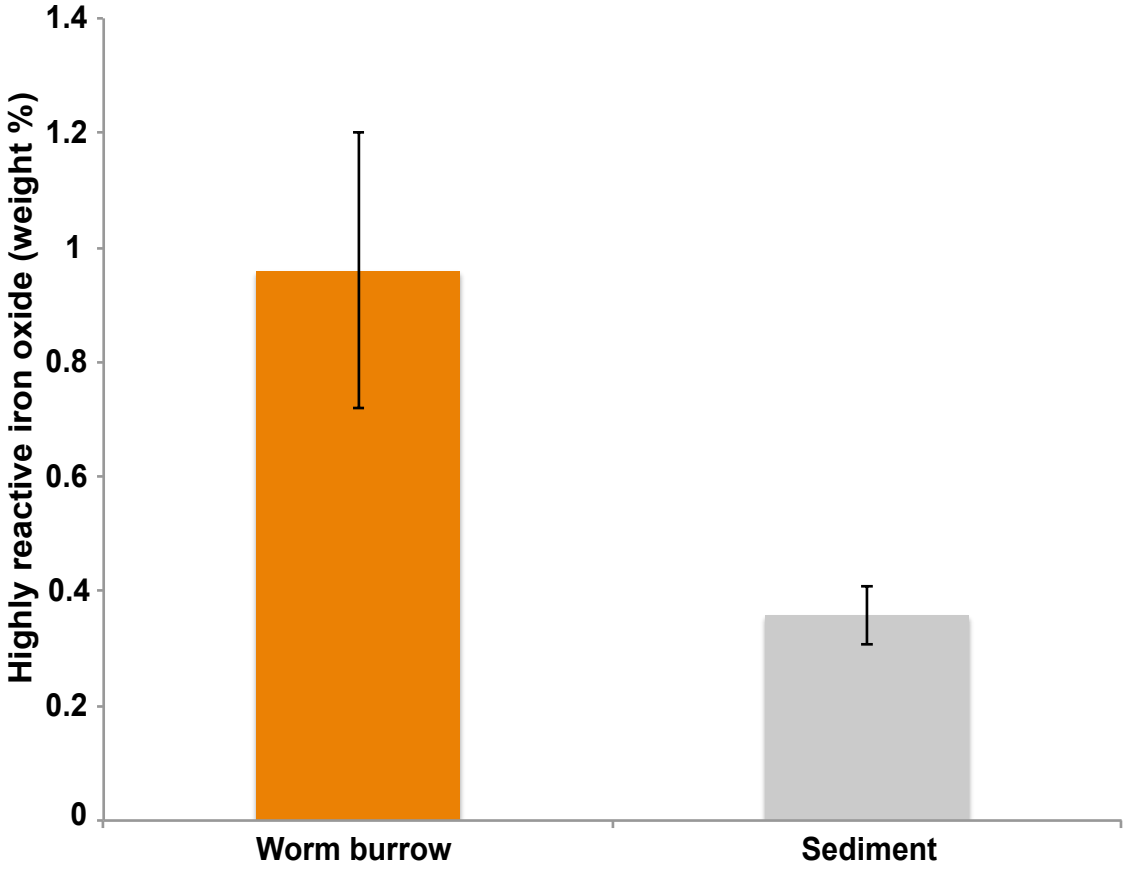
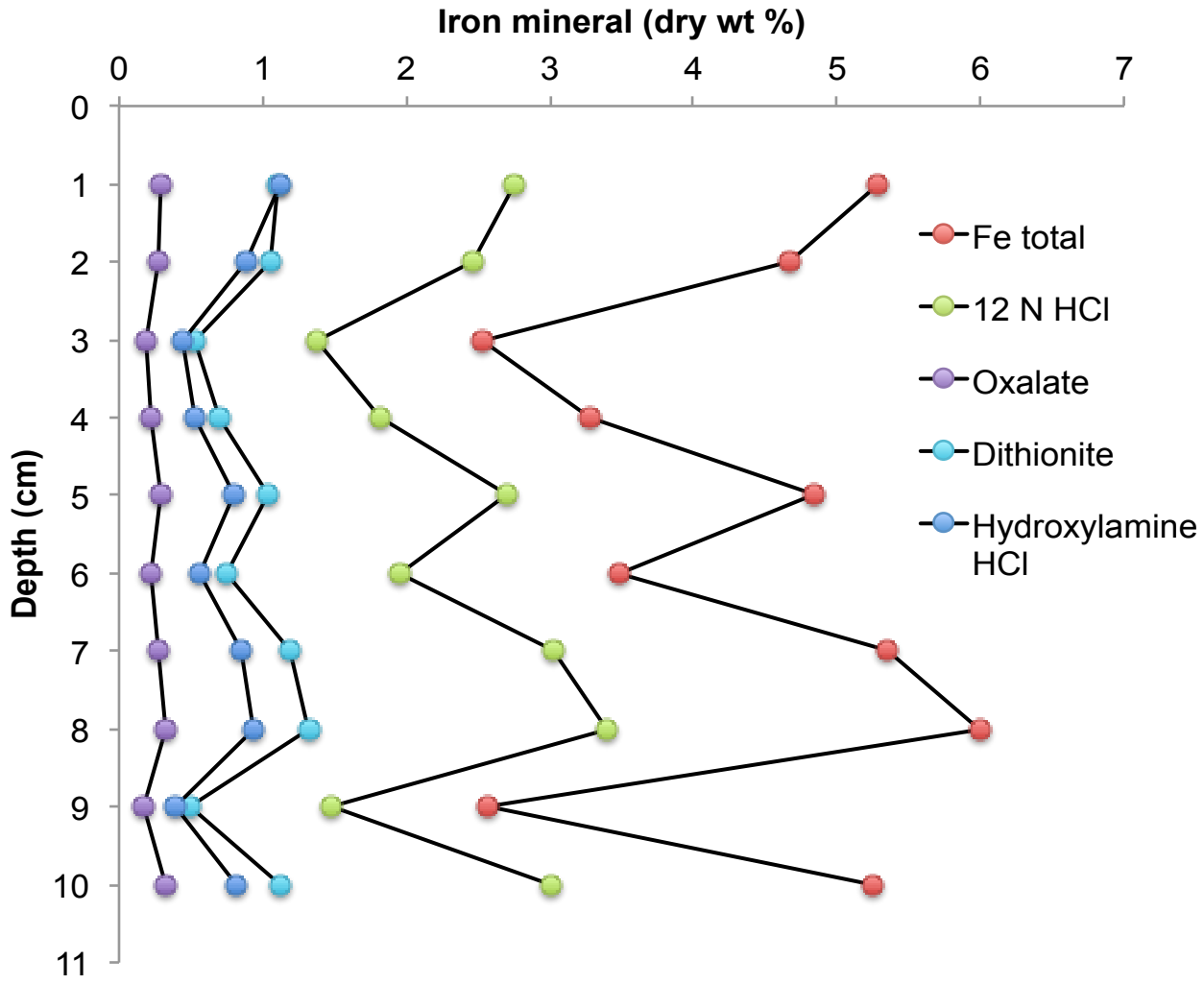


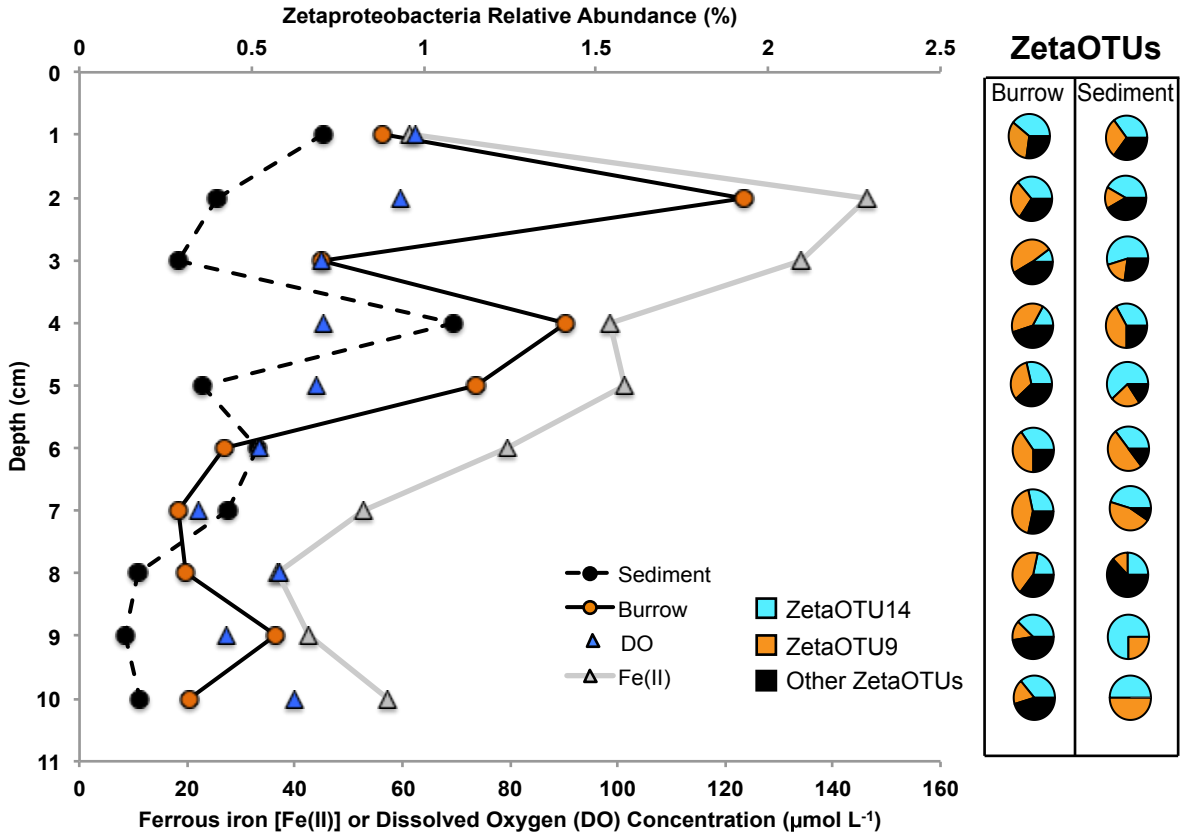
Figure 2



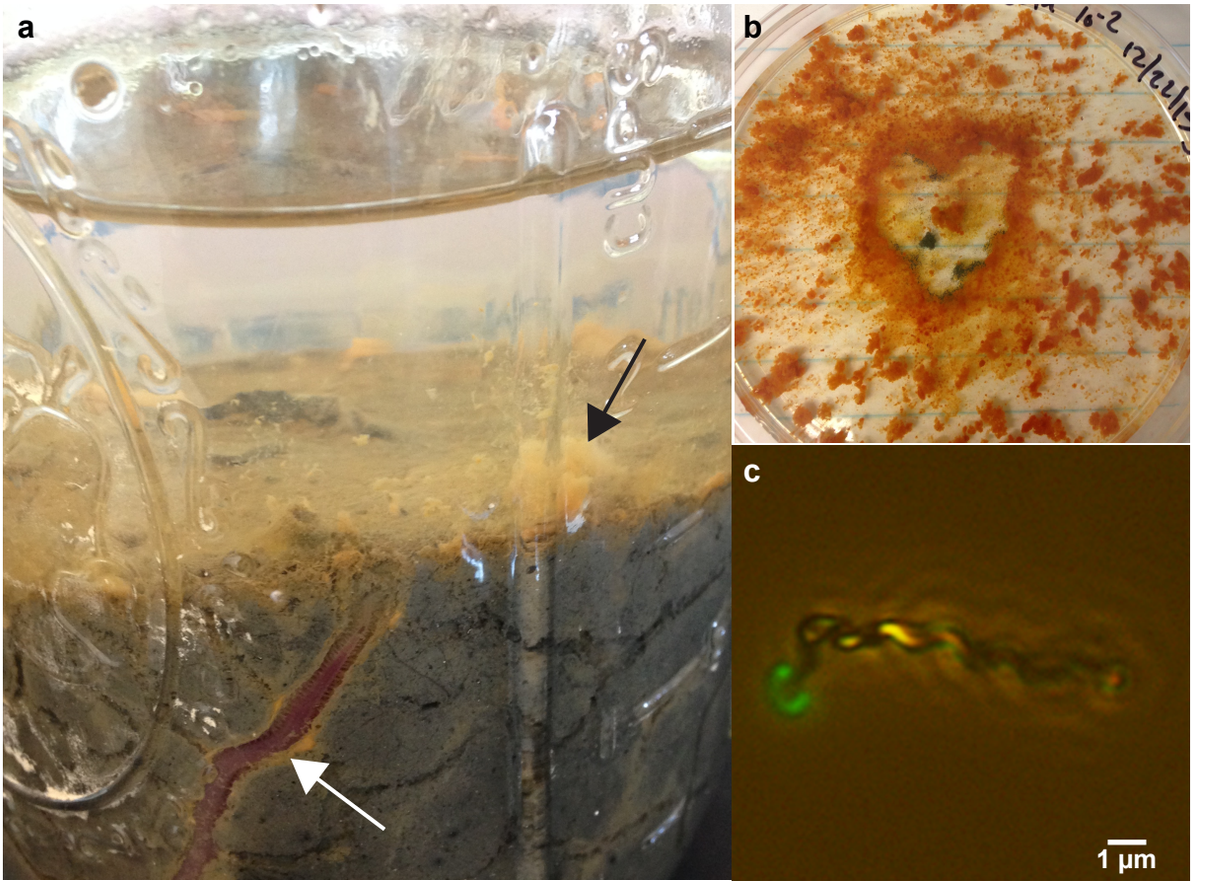
Supplementary Figure S1. Distribution of highly reactive iron oxides from worm burrows (n=4) and sediments (n=4) at a depth of 3 cm from "The Eddy" Sheepscot River, Maine, USA. Iron oxides are enriched at worm burrow walls as compared to bulk sediments. Error bars represent ± 1 standard deviation from the mean. Highly reactive iron oxides were extracted with sodium dithionite for 2 hours (Poulton and Canfield, 2005).



Supplementary Figure S2. Sequential iron mineral extractions from bioturbated sediments at 'The Eddy', Sheepscot River, Maine, USA (sediment temperature= 7 ºC; salinity=35 ppt; 43.994827, -69.648632; collected on 1 March 2016). Poorly-crystalline iron oxides—formed primarily by iron-oxidizing Zetaproteobacteria—comprise about 20 % of the total iron mineral content in these coastal sediments. Note: dithionite extractions are likely overestimating crystalline iron oxides, as dithionite also liberates Fe from iron sulfides, pyrite, and some sheet silicate iron-containing minerals (Poulton and Canfield, 2005). Hydroxylamine HCl=ferrihydrite and lepidocrocite; dithionite=goethite, hematite, and akaganéite; oxalate=magnetite; 12 N HCl=iron silicates; Fe total=sum of all iron mineral phases.



Supplementary Figure S3. Relative abundance of Zetaproteobacteria in iron oxide-lined burrows (orange cirlces and line) and sediments (black circles and line) as a function of depth from "The Eddy" Sheepscot River, Maine, USA (sampled on 1 March 2016). Pore water ferrous iron concentration (grey circles and line, secondary x-axis) and dissolved oxygen (blue triangles, secondary x-axis) as a function of depth is included for geochemical context. Zetaproteobacteria Operational Taxonomic Units (ZetaOTUs) are indicated on the right from burrows and sediments highlighting dominant ZetaOTUs 14 (blue) and 9 (orange).



Supplementary Figure S4. Laboratory bioturbation microcosm (**a**) from a mud flat (Merrow Island) from the Maine coast with abundant iron flocculent (black arrow) forming at the surface of *Glycera dibranchiata* (white arrow) burrows. Zetaproteobacterium strain CSS-1 from ZetaOTU14 growing on zero valent iron (**b**) under low oxygen conditions produces abundant clumps of stalks. Microscope image (**c**) of strain CSS-1 forming an iron oxide stalk stained with Syto13 showing a bean-shaped morphology.

Supplementary Table S2. Representative Zetaproteobacteria operational taxonomic units (ZetaOTUs) clustered at 97 % nucleotide identity across all worm burrow and sediment samples, and isolate, genome, and iron oxide structure characteristics associated with these ZetaOTUs.

ZetaOTU[1]	Abundance (%)[2]	Isolates/SAGs (#)[3]	Biogenic Fe oxide structure[4]	Iron Oxidation Metabolism[5]
ZetaOTU14	31.7	CSS-1/**4**	twisted stalks	cyc_2 (3/4); cbb_3 (4/4); *bd* (3/4)
ZetaOTU9	21.7	TAG1, SV108/**5**	none	cyc_2 (6/7); cbb_3 (4/7); *bd* (3/7)
ZetaOTU23	5.4	GSB2/**0**	twisted stalks	n.d.
ZetaOTU1	5.2	none/**5**	?	cyc_2 (0/5); cbb_3 (2/5); *bd* (3/5)
ZetaOTU4	5.2	none/**2**	?	cyc_2 (0/2); cbb_3 (0/2); *bd* (0/2)
ZetaOTU22	5.1	none	?	n.d.
ZetaOTU31	2.6	none	?	n.d.
ZetaOTU11	1.5	JV1, PV1, M34/**0**	twisted stalks	cyc_2 (3/3); cbb_3 (3/3); *bd* (3/3)
ZetaOTU18	1.2	DIS1, CP-8, *M. micogutta*/**0**	twisted stalks/dreads[6]	cyc_2 (3/3); cbb_3 (3/3); *bd* (3/3)

[1]ZetaOTU classification using ZetaHunter (github.com/mooreryan/ZetaHunter)
[2]Abundance out of total ZetaOTUs
[3]Single-amplified genomes from Lōʻihi and Mid-Atlantic Ridge deep-sea vent sites (Field et al., 2015; Scott et al., 2015; 2017)
[4]?=no structural information available
[5]cyc_2=putative iron oxidase, cbb_3=high affinity C-type heme copper oxygen reductase, *bd*=high-affinity bd-ubiquinol oxygen reductase, n.d.=no data; number in parentheses indicates genes identified out of total isolate genomes/SAGs.
[6]Field et al., 2016