

# Enrichments of Non-phototrophic Sulfur Oxidizing and Sulfate Reducing Bacteria from Salt Pond Sediments

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Microbial Diversity Course 2014. Marine Biological Laboratory

## INTRODUCTION

Microbial mats develop in shallow aquatic environments where sunlight is available forming stratified communities that find their optimal conditions for life along gradients of different chemicals (Seckbach and Oren, 2010). The niche for aerobic organisms is at the top of the mat where oxygenic photosynthesis takes place. Below, purple sulfur bacteria followed by green sulfur bacteria make anoxygenic photosynthesis oxidizing reduced sulfur compounds. Other hemolithotrophic sulfur oxidizing bacteria are located where  $H_2S$  and  $O_2$  are available, and at the bottom, where  $O_2$  is exhausted, sulfate reducers and methanogens are found.

Salt Pond is located next to Oyster Pond, and is connected to the sea through a channel (see supplementary information section). Alongside this channel and next to vegetation, sandy sediments contain stratified communities, yellowish at the top and black at the bottom. Based on the general microbial mat description, the aim of this project was to enrich non-phototrophic sulfur oxidizing and sulfate reducing bacteria from two depths (yellow layer and black layer) and find if there are differences in terms of organisms enriched. In order to compare the communities, CARD-FISH analysis was carried out too.

## METHODOLOGY

### *Sampling and sulfide and oxygen measurements*

Samples from Salt Pond were collected on August 10, 2014. Sediments were divided into two layers for enrichments and CARD-FISH: top layer (first 1 cm depth) and bottom layer (till approximately 5 cm depth). The first layer presented yellowish coloration whereas the second was dark black and had the characteristic odor of sulfide.

Additionally, a core containing the sediments was taken to measure sulfide and oxygen content in the laboratory with 500  $\mu m$  UNISENSE microelectrodes, after approximately 20 hours of exposure to light and to dark to simulate day-night conditions.

### *Enrichment Media*

In order to enrich motile sulfide oxidizers, 10 ml glass tubes were used to make a gradient medium (sulfide-oxygen) based on Nelson and Jannasch (1983). 2 ml of sulfide-plug medium (10 mM sulfide) was dispensed first, and then 6 ml of overlay medium were added (see Supplementary information for media details). After the gradient settled, the tubes were

inoculated with sediment from each layer using a pasteur pipette and kept in the dark for incubation.

Plates containing 5mM sodium thiosulfate were made to enrich thiosulfate oxidizers. All plates contained bromothymol blue to indicate pH change (from blue to yellow as it acidifies). As inocula, 100 µl of sediment pore water were used in triplicate for each layer. In order to try two different O<sub>2</sub> conditions, half of the plates were incubated on the lab bench and the other half in an anaerobic jar with an Anaerobe pack inside.

In order to enrich sulfate reducers, base medium was prepared and then aliquoted into the serum bottles in the anaerobic chamber (see supplementary information for media details). Four different electron donors were used separately: 10 mM acetate, 10 mM lactate, 20 mM methanol and H<sub>2</sub> (80% H<sub>2</sub>, 20% CO<sub>2</sub> gassing). Pea-sized amounts of sediment from the two layers were used as separate sources of inocula.

### ***Sulfide and acetate determinations***

Sulfide measurements were made using the HACH Test kit for hydrogen sulfide that is based on the spectrophotometric assay developed by Cline (1969). 500 µl of sample from the serum bottles for sulfate reducers were fixed with 1 ml zinc acetate and then diluted as needed and 20 µl of the reactant 1 and then the reactant 2 were added. The absorbance at 670 nm was measured in the spectrophotometer.

For acetate measurement on HPLC, 900 µl of sample were fixed with 100 µl of sulfuric acid 5N, and filtrated with 0.2 µm filter.

### ***CARD-FISH***

The sediments used to enrich sulfur bacteria were also used for CARD-FISH analysis. 1 ml of sediment from each layer was fixed with 9 ml of 4% PFA (3.6% final concentration) for 22hs at 4°C. After washing and then storing with 1ml ethanol-PBS (1:1 ratio), samples were sonicated 7 times for 30 seconds with a 30 second break in between with 1 pulse per second and an intensity of around 20%. 500 µl of the sample were diluted 40 times with 1X PBS and vacuum-filtered on a Millipore GTTP membrane filter with 0.2µm pore size.

Filters were embedded with 0.4% low melting agarose and dried at 37°C and then cut into slices. Each slice was permeabilized with 100000/mL lysozyme (in 0.05 M EDTA, 0.1 M Tris-HCl) and incubated at 37°C for 90minutes. The slide was placed into a petri dish, washed twice with MilliQ and dried at 37°C. Internal peroxidases were inactivated with 0.01 M HCl for 1 minute at room temperature, washed in 1X PBS and then incubated in 3% H<sub>2</sub>O<sub>2</sub> in MilliQ water at room temperature, and then washed with MilliQ water and air dried on a whatman paper.

1 µl of probe solution (50 ng/µL) was added to 300 µL of hybridization buffer and hybridization was performed in a 1.5 ml eppendorf tube for 3 hs at 47 °C. Slides were washed in prewarmed washing buffer at 48°C for 10 minutes before being washed twice in PBS.

10 µL of 0.15% H<sub>2</sub>O<sub>2</sub> in PBS and 1 µL of AlexaFluor594-tyramide conjugate (1 mg dye/mL) was added to 990 µL of amplification buffer in a 1.5 ml eppendorf tube and the slices were dipped in. Amplification took place for 30 minutes at 46 °C.

Slides were washed in 1x PBS for 10 minutes, then 3 times in MilliQ water, and dried in the dark. Next, the slices were mounted with 4:1 Citifluor:Vectashield containing 1  $\mu\text{g}/\text{mL}$  DAPI and analyzed with the epifluorescence microscope.

Probes selected for FISH analysis were EUB338-I-III, CYA664, GAM42a (with competitor BET42a unlab), DELTA495 a-c (with competitors), PSB PiBe461, JTB255, EPS914 and NON338.

## RESULTS

### Sulfide and Oxygen profiles

Microelectrodes measurements revealed that sulfide and oxygen content in depth followed a profile expected for a microbial mat, where oxygen saturation is found at the top due to oxygenic photosynthesis during the day, and sulfide increases with depth since sulfate reduction at the very bottom is producing sulfide, whereas at night, oxygen decreases and sulfide diffuses towards the surface (Visscher et al., 2002). In the core taken from Salt Pond sediments after light exposure, oxygen reached high levels (more than 600  $\mu\text{mol}/\text{l}$ ) in the first cm from the sediment surface. Around 2 cm deep, sulfide started to increase till it reached around 14  $\mu\text{mol}/\text{l}$  at 3.5 cm. The pH also dropped from 8 to 7 on average at 1 cm depth. After dark exposure, the oxygen level decreased and diffused completely in the top 0.5 cm of the sediment, while sulfide reached around 10  $\mu\text{mol}/\text{l}$  at 4 cm depth. The sulfide profile shifted closer to the surface (till around 1cm depth) in comparison with the profile of the sediment that was exposed to light (Fig.1).

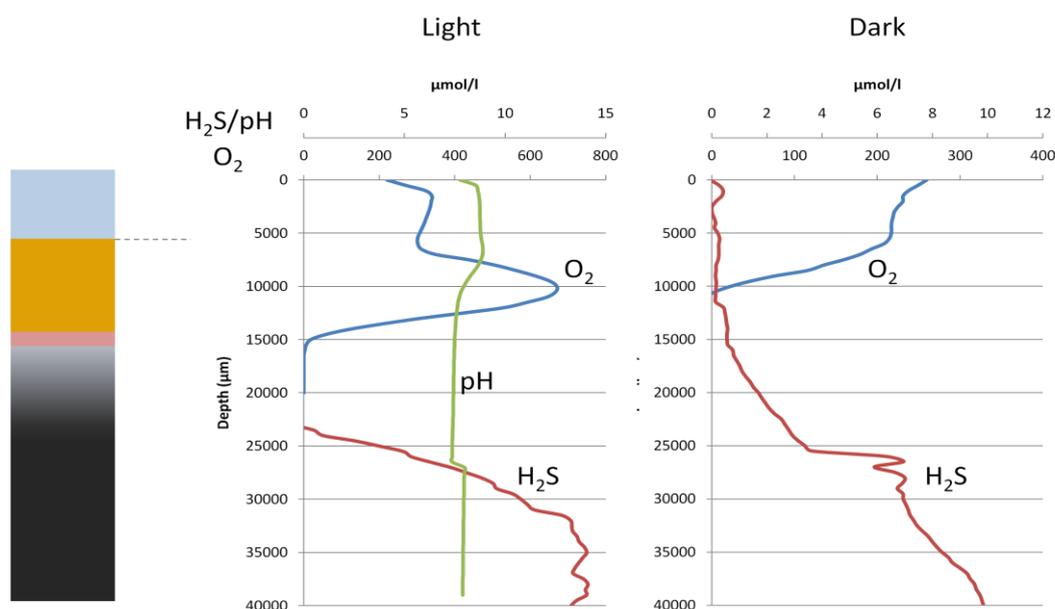
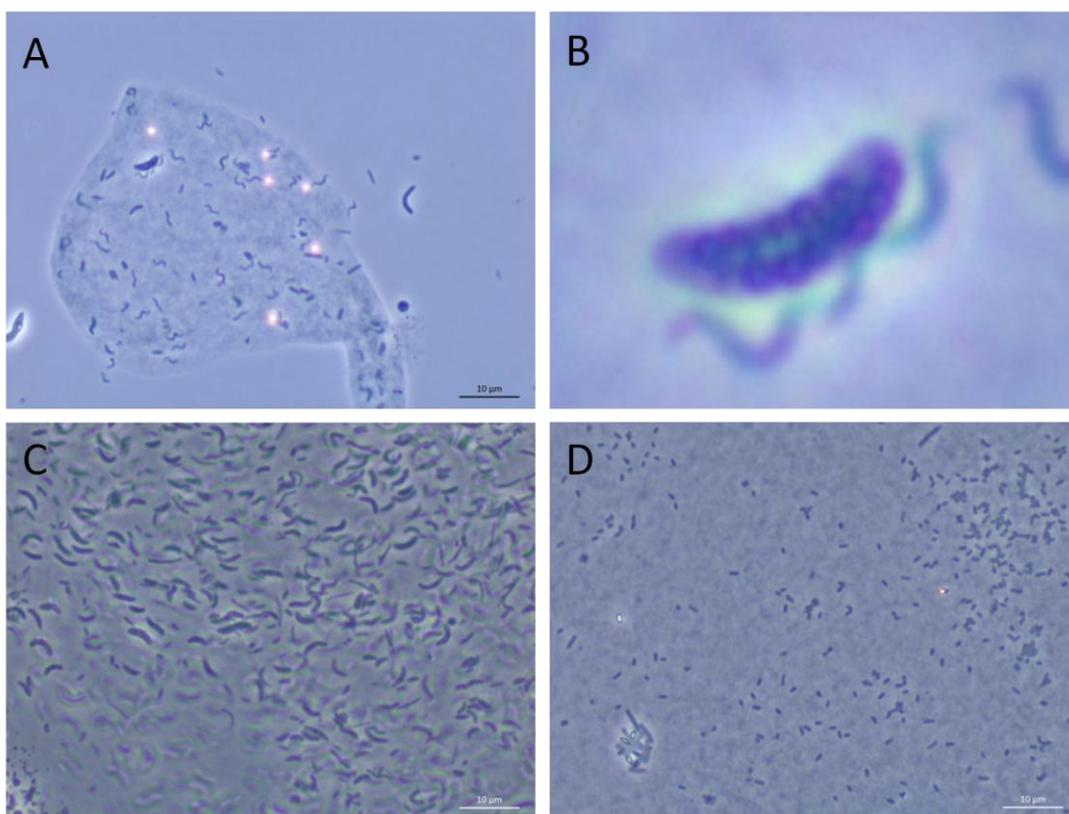


Fig. 1. Oxygen, pH and sulfide profiles measured in sediment core from Salt Pond. The illustration in the left shows in light blue the water above the sediments, in yellow the first sampled layer and in black the second one.

### Sulfide oxidizing bacteria enrichments in gradient tubes

After 7 days of incubation, a thick whitish plate developed at the top of the gradient tubes, in between oxygen (noticed by the pink coloration of the resazurin at the very top) and sulfide (the media looked transparent below the mentioned plate), indicating growth. Distinct morphotypes were observed under the microscope. Those enrichments inoculated with sediment from the top layer, showed long rod, vibrio and pea-like shaped organisms. Sulfur

granules were evident in the media and inside the cells (Fig. 2, A-B). In the case of tubes inoculated with sample from the bottom layer, similar morphotypes and sulfur inclusions were observed (Fig. 2, C-D).



**Fig. 2.** Photomicrographs of the growth plate developed in the gradient tubes. A-B) From tubes inoculated with sample from the top layer and C-D) bottom layer.

### Thiosulfate plates

After 7 days of inoculation, bromothymol blue changed its coloration as follows: those incubated in the anaerobic jar (micro-oxic conditions) turned yellowish, including the control plate, indicating that pH decreased as a consequence of CO<sub>2</sub> released by the anaerobic pack. For those plates incubated under fully oxic conditions, a change in color was more evident in those plates inoculated with samples from the bottom layer, suggesting that growth might have acidified the media (data not shown).

Distinct colonies were noticed under the dissecting scope, and were streaked in new plates. Those from the top layer under fully-oxic conditions showed whitish colonies (Fig.3, A) and one of them seemed to acidify the media (Fig.3, B). For those from the bottom layer under fully-oxic conditions, rare structures were noticed under the dissecting scope. One of them has precipitates (Fig. 3, C) whose composition did not reveal S as a main component with the SEM-EDS analysis (data not shown). Additionally, some colonies had a different appearance, some were yellow and seemed to contain granules (Fig. 3, D-F) and others were white and had the appearance of filamentous precipitates (Fig. 3, E). The chemical composition of these structures was not revealed.

Finally, those plates under micro-oxic conditions showed similar yellow colonies as those under fully-oxic conditions (Fig.3, G-J). That morphology was more abundant in plates

inoculated with the bottom layer, and only one colony from the top layer showed the same appearance (Fig. 3, G). To figure out the elemental content, SEM-EDS analysis was carried out only for one colony (Fig.3, H) due to short time available. S was the main element in high proportions.

The presence of sulfur in those colonies presents a dilemma, because sulfur precipitation as a result of thiosulfate oxidation was reported only under anoxic conditions. In order for this to happen, this reaction may take place:  $S_2O_3^{2-} + 1/2 O_2 \rightarrow S_0 + SO_4^{2-}$ . Thermodyn analysis revealed that this reaction is thermodynamically favorable (data not shown). More analysis should be done to confirm the presence of sulfur in colonies under micro-oxic and oxic conditions, and to elucidate its biological origin.

### **Sulfate reducers enrichments**

Serum bottles showed turbidity in those cases where lactate and methanol were used as electron donors. Microscopy showed a prevalence of coccoid-shaped microorganisms in enrichments inoculated with top layer sediment (Fig. 4, A-B); whereas there was no morphotype predominating enrichments with bottom layer inocula (Fig.4, C-D).

Sulfide production indicated sulfate reduction in all the enrichments. There was a high production of sulfide in those bottles where  $H_2$  was the electron donor, followed by lactate bottles (Fig.5, A-B).

As expected, acetate concentrations decreased, due to oxidation of this electron donor. At the same time, sulfide increased due to the reduction of sulfate (Fig. 5, C-D). Nevertheless, the stoichiometry is unbalanced, probably because measurements were not accurate enough, and the enrichments with environmental inocula may contain reduced carbon compounds that were not measured and that may be electron donors as well.

### **CARD-FISH**

CARD-FISH filters contained few cells, especially those from the bottom layer. The analysis revealed the presence of Cyanobacteria and purple sulfur bacteria in both layers, top (Fig. 6) and bottom (data not shown). Epsilon, Gamma and Delta-proteobacteria and uncultured Gammaproteobacteria (probe JTB255) were only found in the top layer (Fig.7). However, they might be present in the bottom layer as well and might have been missed due to low cell content.

### **CONCLUSIONS**

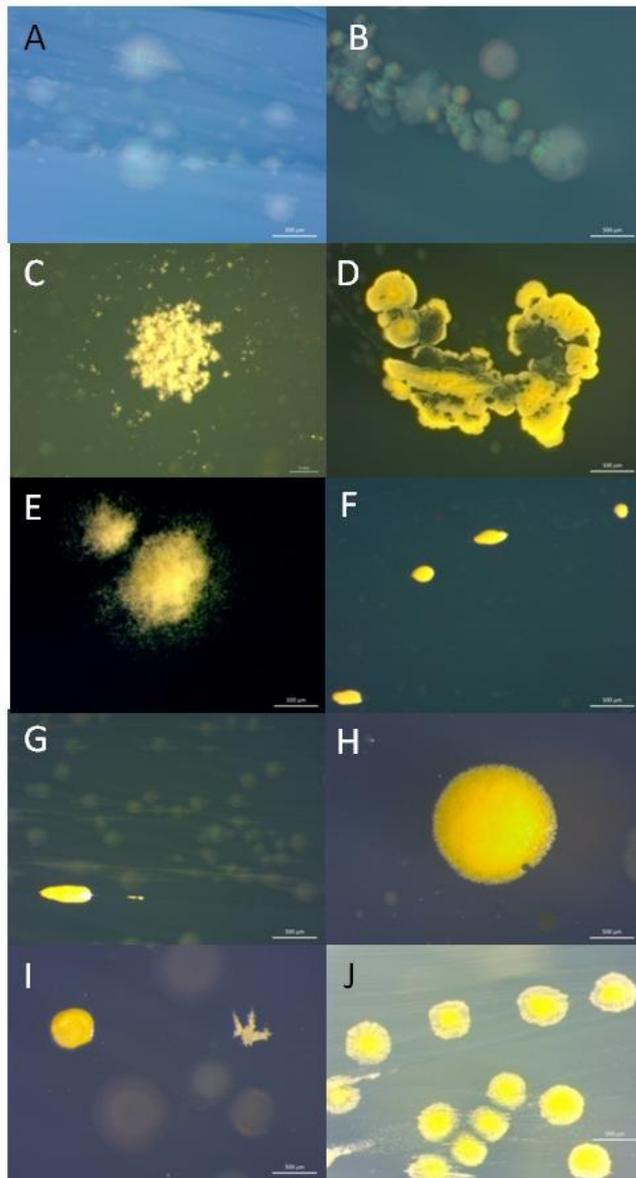
Oxygen and sulfide profiles showed that there is an active bacterial community developing in the sediments from Salt Pond.

There were no remarkable differences in terms of sulfide oxidizing bacteria enriched from the top and the bottom layer.

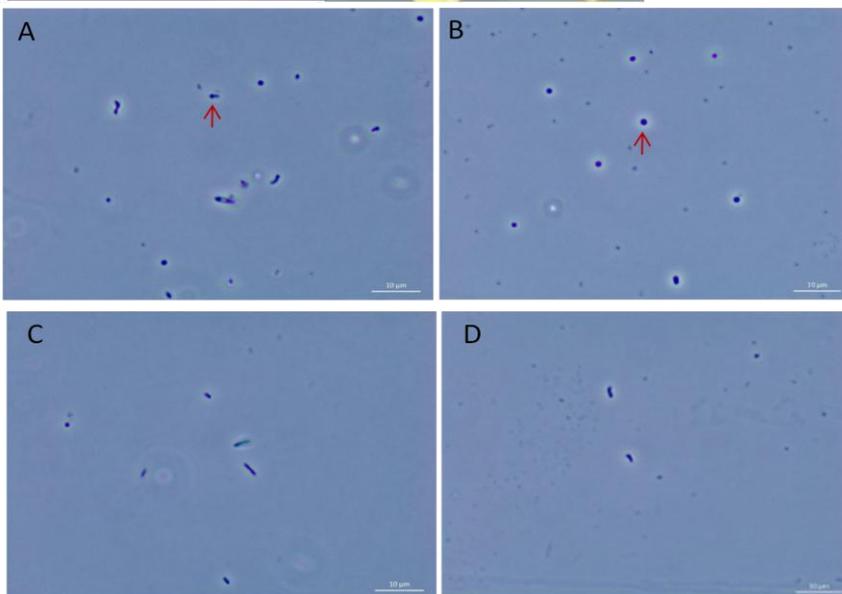
Results from the thiosulfate plates suggested the possibility of sulfur production from biological thiosulfate oxidation under oxic and micro-oxic conditions. A greater variety of colonies and precipitates were found in the bottom layer.

Enrichments for sulfate reducers resulted in the prevalence of a coccoid-morphotype in the top layer.

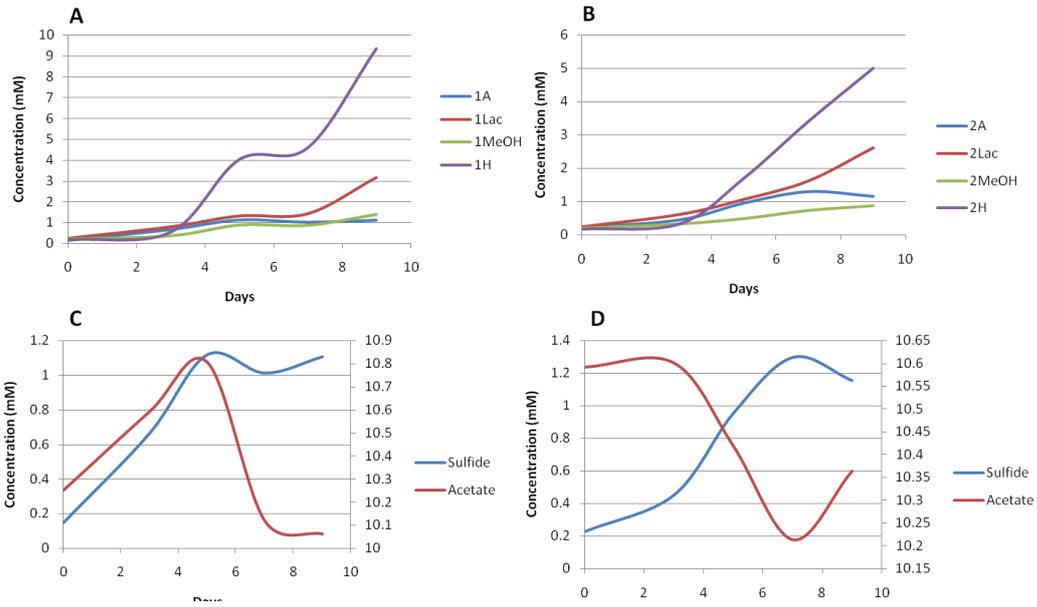
Qualitative CARD-FISH showed low cell content in the bottom layer, and the presence of purple sulfur bacteria and sulfate reducing taxonomic groups in the sediments.



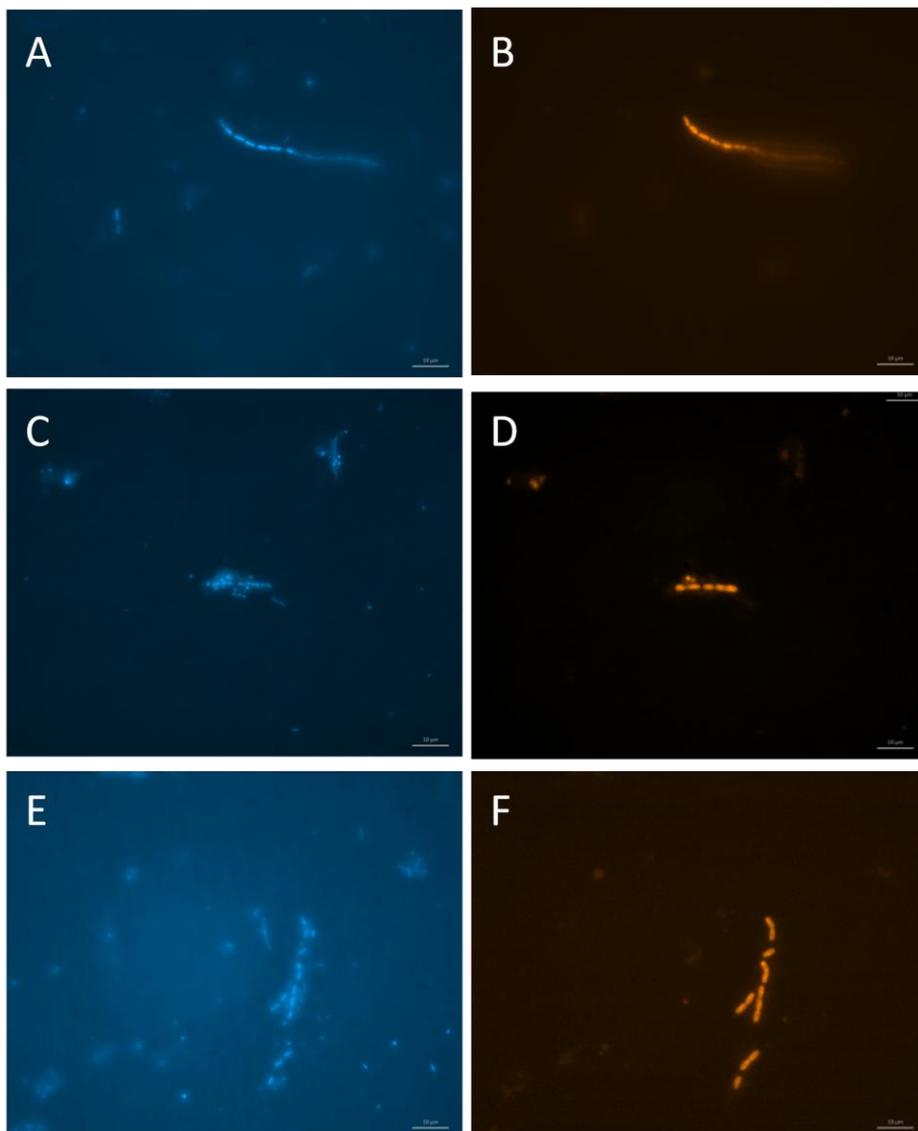
**Fig. 3.** Colonies observed in thiosulfate plates inoculated with sample from the top layer (A-B) and bottom layer (C-F) under fully-oxic conditions. Colonies observed in plates inoculated with sample from the top layer (G) and bottom layer (H-J) under micro-oxic conditions.



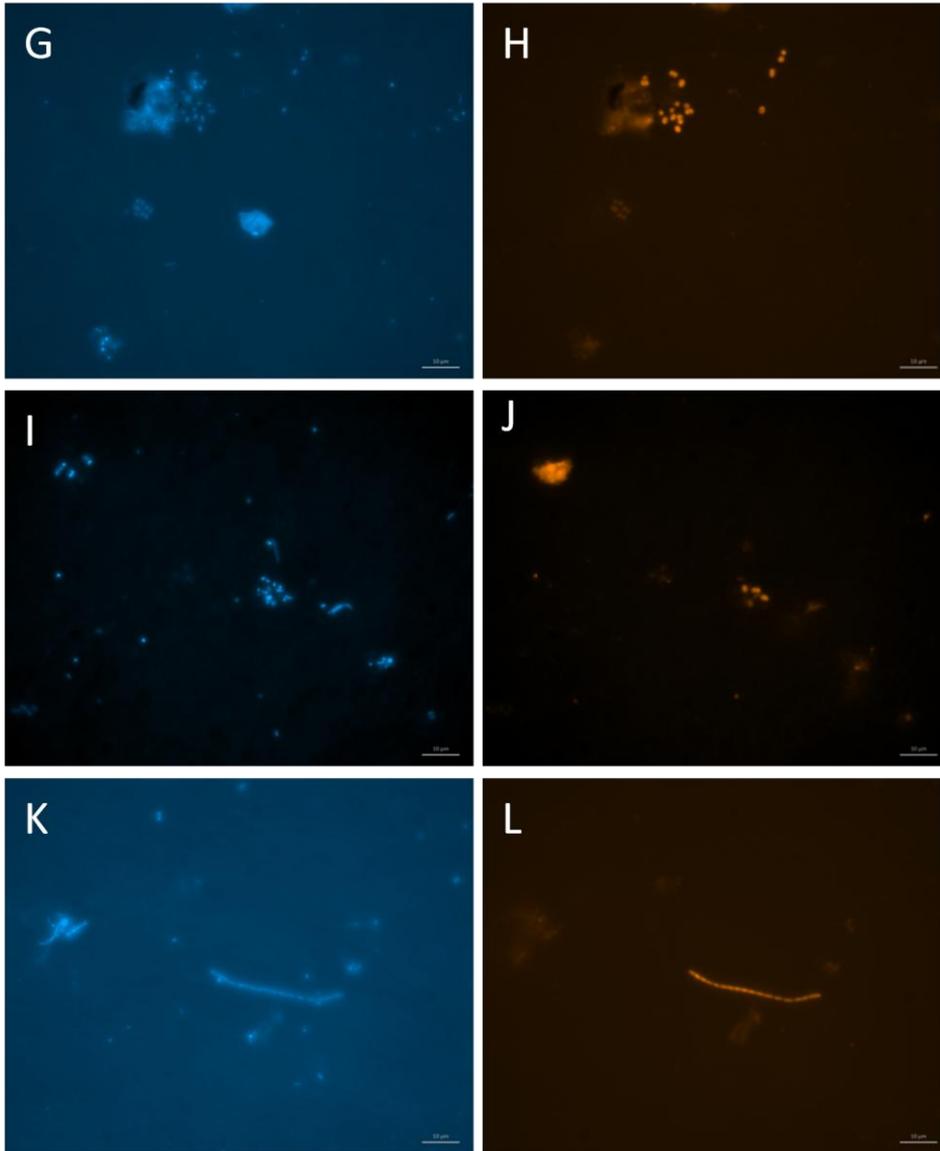
**Fig. 4.** Photomicrographs of sulfate reducers enrichments A) lactate as the electron donor and top layer as inoculum, B) methanol as electron donor and top layer as inoculum, C) lactate as electron donor and bottom layer as inoculum, D) methanol as electron donor and bottom layer as inoculum.



**Fig. 5.** Sulfide measurements of sulfate enrichments with top layer (A) and bottom layer (B) as inoculum. Acetate measurements from top layer (C) and bottom layer (D) sulfate reducers enrichment with acetate as electron donor.



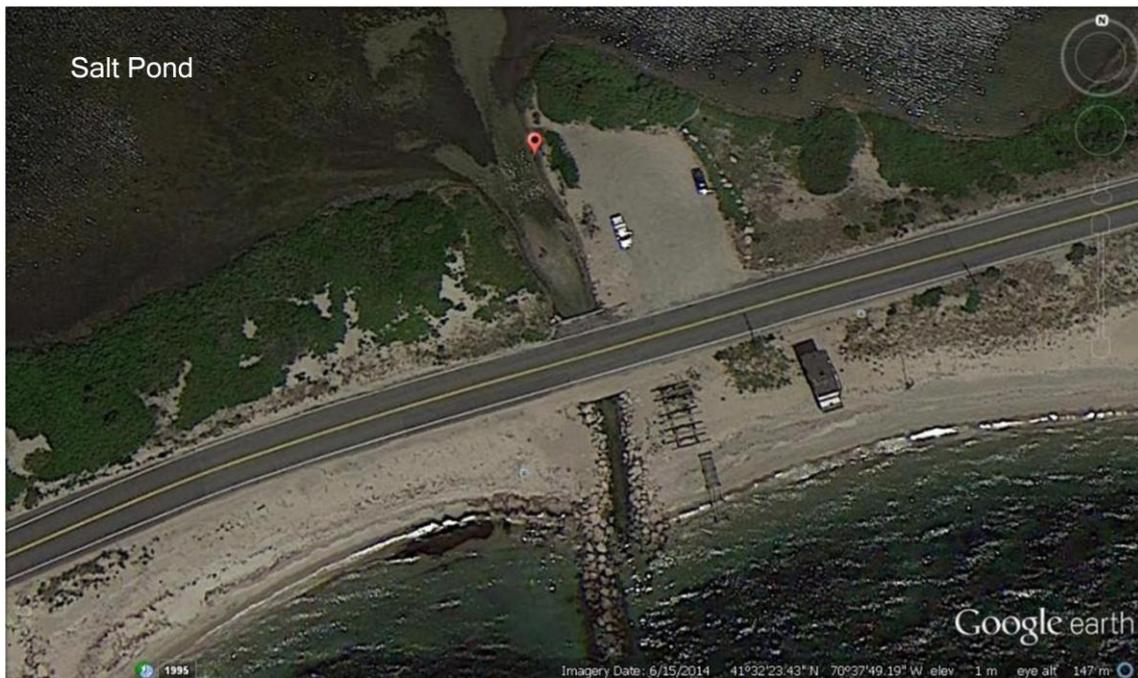
**Fig. 6.** CARD-FISH epifluorescence microscopy images. A, C, E) DAPI-stain. B) Cyanobacteria signal from sample from top layer. D) PSB PiBe 461 signal for sample from top layer. F) Epsilon-proteobacteria signal from sample from top layer.



**Fig. 7. CARD-FISH epifluorescence microscopy images. G, I, K) DAPI-stain. H) Gamma-proteobacteria signal from sample from top layer. J) Delta-proteobacteria signal for sample from top layer. L) JTB255 signal from sample from top layer.**

## SUPPLEMENTARY INFORMATION

### Sampling Location



#### Gradient tubes media

Sulfide plug contained per 100 ml:

100 ml Water base (NaCl 195.5 mM, MgCl<sub>2</sub>·6H<sub>2</sub>O 8.46 mM, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.57 mM and KCl 3.83 mM).

ml 100x NH<sub>4</sub>Cl solution

ml 100x K phosphate solution

1% resazurin solution

1.5 g purified agar

1 ml 1M MOPS buffer, Ph 7.2

Autoclave

5 ml 200 mM sterile sodium sulfide solution

0.2 ml 1M sterile sodium bicarbonate

Overlay medium per 400 ml:

400 ml Water base (NaCl 195.5 mM, MgCl<sub>2</sub>·6H<sub>2</sub>O 8.46 mM, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.57 mM and KCl 3.83 mM).

0.4 ml 100x NH<sub>4</sub>Cl

0.04 ml 100x K phosphate solution

40 µl 1% resazurin solution

0.4 g gelrite

4 ml MOPS buffer, pH 7.2

Autoclave

0.8 ml 1M sterile sodium bicarbonate

0.4 ml sterile 13-vitamins stock

0.4 ml sterile trace metal solution

**Thiosulfate plates medium (per liter):**

Water base (NaCl 195.5 mM, MgCl<sub>2</sub>·6H<sub>2</sub>O 8.46 mM, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.57 mM and KCl 3.83 mM).

1 ml 1M sodium sulfate

1.25 g/L sodium thiosulfate (5 mM)

1 ml/L NH<sub>4</sub>Cl 100X

0.1 ml/L K phosphate, pH 7.2 100X

15g/L agar

0.02 g/L bromothymol blue

20 ml of 1M MOPS buffer pH 7.2 (10 mM final)

Autoclave

10 ml of 1M sodium bicarbonate (10mM final)

1ml of 1000x vitamin solution

1ml of 1000x trace element solution

**Sulfate reducers medium**

~1 liter 1x SW-Base (Some loss of volume occurs due to boiling)

5 ml 1 M NH<sub>4</sub>Cl solution

10 ml 100 mM K phos, pH 7.2

5 ml 1 M MOPS Buffer, pH 7.2

1 ml Trace elements

100 µl 1% Resazurin

-Boil 1 L for 10 minutes in 3 L round bottom flasks under N<sub>2</sub>/CO<sub>2</sub> (80%:20%). Cool under stream of N<sub>2</sub>/CO<sub>2</sub> gas.

1ml Multivitamin solution

70 ml 1M Sodium Bicarbonate

**References**

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