Investigating on microbial community of Trunk River shore sediment core profile by CARD-FISH and qPCR

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Abstract

Trunk River shore sediment has been characterized as the "rainbow sediment" due to the clear color transition from the surface layer to lower layer. And the microbial community composition within the depth profile should be responsible for color change, which has been long considered as the general conception, yet, still lacking of quantitative microbial community data support. In this study, 6 depth layers in the depth profile from "rainbow sediment" have been subjected to CARD-FISH and DAPI staining on 5 major microbial groups. The transitionary distribution of cyanobacteria, Delta-, Gamma-proteobacteria, Bacteroidetes and archaea in the depth profile have been investigated. Cyanobacteria showed a decreasing trend in abundant ratios within total microbial community along the depth profile. Deltaproteobacteria was co-abundant with the sulfide distribution. Additional, qPCR on quantifying the cell density is accordant with that of DAPI counting, suggesting the high reliability of cell quantification results. The draft conclusion of correlation relationship between the major microbial group distribution pattern and physicochemical profiles could further facilitate the decision of choosing sediment types to conduct microbial enrichment.

Introduction

Trunk River is the outlet of Trunk Pond (Woods Hole, MA) to the east coast of Cape Code. The water salinity goes gradiently down from the Trunk Pond to the mouth of Trunk River, and Trunk Pond also contains a thick layer of decaying organic harboring high level of sulfide. Shore sediment of Trunk River has been described as the "rainbow sediment" due to the clear color transition from the surface layer to around 5-10 cm depth layer. The top brown layer is believed to be composed of both cyanobacteria and diatom groups. The lower green layer is believed to be dominated by cyanobacteria. At the following violet and black layers from around 0.5 cm to 5-10 cm deep, the local microniches will be mainly characterized as sulfide rich and anoxic, and many anoxygenic phototrophs, such as purple non-sulfur bacteria (PNSB), purple sulfur

bacteria (PSB), green sulfur bacteria (GSB) could be present in these upper layers, where light intensity is still high and a number of electron donors for their anaerobically phototrophic growth are available, such as sulfide, thiosulfate and etc. PSB belong to Gammaproteobacteria, while PNSB are represented by a diversity of Alpha- and Beta-proteobacteria. GSB are mostly represented by Chlorobi.

The general conception of the differentiation of major microbial groups along the depth profile of "rainbow sediment" is prevalent for many years, while, lacking for any qualitative or quantitative evidence. The relationship of microbial community composition of the rainbow sediment at different layers and physiochemical parameters has seldom been addressed. The hypothesis that light availability on the top layer will benefit the growth of cyanobacteria, and relative high sulfide amount, oxygen level in the lower layers will support the growth of anoxygenic phototrophs still remains to be testified. Classifying of the microbial community composition of "rainbow sediment" quantitatively could help to address the long-time concept conclusively and provide more insight into how physicochemical parameters along the depth profile influence the distribution of microbial communities.

CARD-FISH (catalyzed reporter deposition-FISH) is famous advanced new edition of FISH to overcome the shortage of normal FISH on insufficient sensitivity and low permeability of cells (Kubota 2013). In CARD-FISH, probes are tagged with horse radish peroxidase (HRP). After, hybridization, excess probes are washed out. And at the presence of hydrogen peroxide and fluorescently labeled tyramide, HRP will convert tyramide into the radical intermediate and fluorescence will be active. Since that a single HRP molecule could convert multiple fluorescently labeled tyramide into radical intermediates, the hybridizing signals will be amplified (Kubota 2013). This will significantly increase the sensitivity and reduce the background interference. CARD-FISH probes targeting on major microbial groups, such as Archaea, Cyanobacteria, Deltaproteobacteria, Gammaproteobacteria, Bacteriodetes (Cytophaga/Flavobacteria) and DAPI staining targeting on all microbial nucleus DNA was used in this study to measure the cell quantities of these major microbial groups in each layer of the depth profile. At the same time, qPCR was also used to quantitatively measure the 16S rRNA gene quantities of all prokaryotes by using the prok-universal primer pair, aiming to compare the difference of these two methods.

Methods

Sampling

Sediment core was taken back by 60 ml syringe with plungers sealing up the top and bottom, and transferred to lab soon after sampling from Trunk River shore, Falmouth, MA (41°32'05.1"N 70°38'30.5"W). There are plenty of sands and little rocks in the shore of Trunk River, and in order to acquire intact and practical core samples for experiment, especially for microsensor measurement, sampling sites close to the interface of water and shore with fewer rocks are recommended. From the top to bottom,

6 layers of sediments with 0.5 cm thickness were acquired to conduct the following CARD-FISH and DNA isolation. Layer 1-6 are within 0-0.5, 0.5-1.0, 1.75-2.25, 2.75-3.25, 3.75-4.25, 4.75-5.25 cm depth, respectively.

Microsensoring

Four sensors were applied in this study, including the amperometric sensors for O_2 and H_2S, which directly produce an electrical current as a function of the analyte concentration; and potentiometric sensors for pH and redox potential, which will create a voltage signal in response to an analyte and need a reference electrode as the reference in the same sample core. For O_2, H_2S, and redox potential, sediment core with depth 0-7 cm, while, for pH, sediment core with depth 0-4 cm, were measured. Details are in the protocol.

DNA isolation and quantity measurement

0.25-0.3 g wet soil sediment was used to isolate DNA by PowerFecal® DNA Isolation Kit (MOBIO) according to manufacturer's instruction. DNA quantities were measured by Quantus Fluorometer (Promega) according to manufacturer's instruction.

qPCR

I *Standard plasmid construction*

The standard plasmid was constructed by inserting the 16S rRNA gene fragments amplified from *E. coli* β2155 (466 bp, residues between 331-797). The bacterial universal PCR primers were: Universal-F, 5'-TCCTACGGGAGGCAGCAGT-3' (Tm, 59.4°C) and Universal-R, 5'-GGACTACCAGGGTATCTAATCCTGTT-3' (Tm, 69.9°C) (Nadkarni et al. 2002). The PCR thermocycling setting was preheating at 95°C for 2 min; 25 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extending at 72°C for 30s; and final extending at 72°C for 10 min. The 20μl PCR mixture contained: 10μl 2×GoTaq® Green Master Mix (Promega), 1 μl each forward and reverse primer (conc. 10 ng/ μl), 2 μl *E. coli* genomic DNA (boil *E. coli* in 50 μl ddH_2O) and ddH_2O to fill up the total volume to 20μl. The PCR products were harvested and purified by Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacter's instruction. The standard plasmid with *E. coli* 16S rRNA gene fragment was made by pGEM®-T Easy Vector Systems according to manufacter's instruction. High concentration of standard plasmid was achieved by transforming it into BL21 competent *E. coli* which will be selected by ampicillin resistance and harvesting plasmids by Wizard® Plus SV Minipreps DNA Purification System (Promega). Finally, standard plasmid concentration was quantified by Quantus Fluorometer (Promega), and 10 times gradiently diluted standard plasmids were made. Abundance in gene copy number/μl = (amount/μl$\times 6.022\times 10^{23}$)/(length$\times 1\times 10^{9}\times 660$). Here, plasmid length was 3466 bp (3000bp, pGEM-T; 466bp, inserted fragment). The high plasmid concentration is 7.2 ng/μl. So the calculated plasmid copies/μl equals to 1.9×10^{9} copies/μl.

II *Quantitative PCR*

Triple replicates for 6 DNA samples, 5 gradiently diluted standard plasmids and 1 negative control (nuclease free H_2O) were applied to conduct qPCR. qPCR reaction mix contains: 10 µl GoTaq® qPCR Master Mix (Promega), 1 µl each Universal-F/-R (10 mM), 1 µl template (0-5 ng/µl DNA), nuclease free H_2O (fill up to 20 µl). Two step method was applied with the setting as: 15s at 95°C, 60s at 60°C for 40 cycles. After qPCR, the standard curve was generated with r^2=0.9987, Eff%=71.99%.

DAPI and CARD-FISH counting

For each layer, CARD-FISH probes and DAPI were applied on 7 slices, including the positive and negative controls (Table 1). The microscopic images under 100× oil lens by Zeiss Axio Imager 2. The filtering wavelength for Alexa-488 tyramide is 509 nm (EGFP channel), and that for DAPI is 465 nm (DAPI channel). In individual microscopic field, CARD-FISH probe and DAPI counts were obtained, and densities were calculated accordingly. And the average density of CARD-FISH for each layer's filter were obtained by 5 experimental slices. Statistics were conducted in Excel. Wet weights of sediments which were used for CARD-FISH were recorded, and the cell number/g sediment for each layer was calculated accordingly.

Table 1. CARD-FISH probes applied in this study

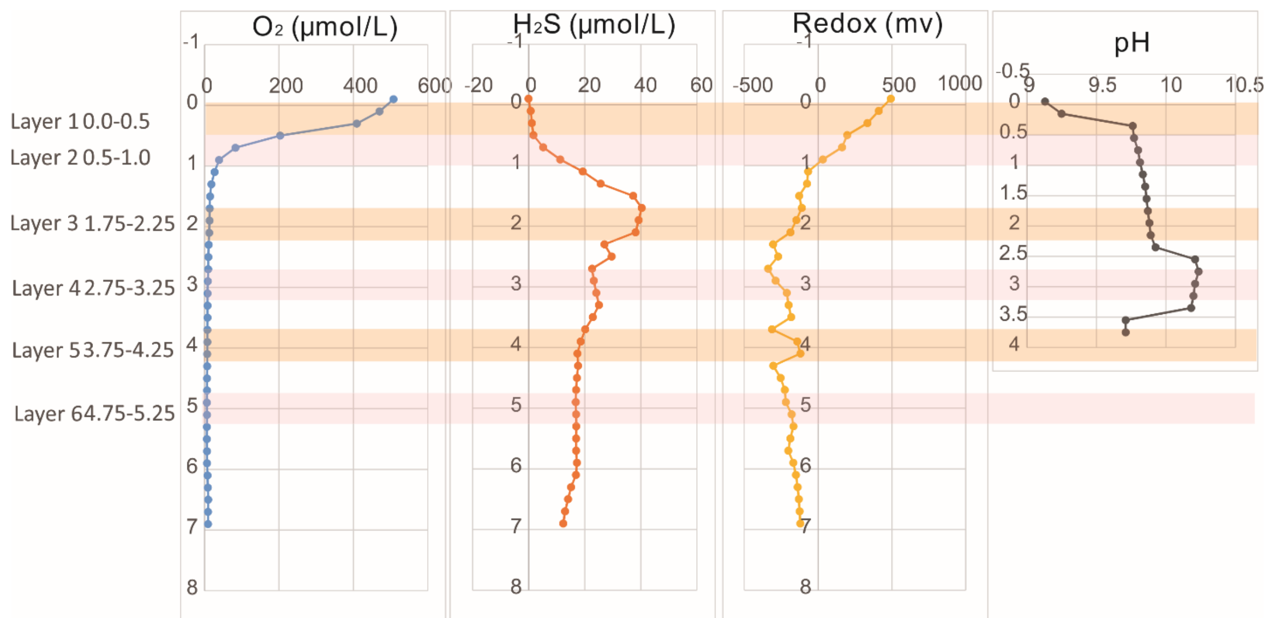
Tag	***Probes***	***Targets***
A	ARCH915	Archaea
B	CF319a	Cytophaga/Flavobacteria (Bacteriodetes)
C	CYA664	Cyanobacteria
D	DELTA495a-c (competitor DELTA495a-c w/o label)	Deltaproteobacteria
E	EUB338-I-III	Eubacteria (+)
F	GAM42a (BET42a w/o label)	Gammaproteobacteria
G	NON338	Nonsense (-)

Results

Microsensoring results

Oxygen concentration drastically dropped from 500 µmol/L to around 10 µmol/L in the top 1 cm depth. H_2S concentration initially increased from nearly 0 µmol/L to around 40 µmol/L in the top 2 cm depth, and went down to the level around 20 µmol/L in the following depth layers. Redox potential was dropping down from 500 mv to 0 mv in the top 1 cm, keeping negative in the following depth layer. The lowest redox potential points appeared within 2-4 cm around -300 mv, however, there were still some fluctuations in this region, e.g. a peak with around -100 mv appearing at 4 cm depth point. The pH value profile along the sediment core varied from around 9.1 to 10.3. There was a drastic increasing in the top 0.5 cm layer of pH value, from around 9.1 to 9.7; and then, pH profile increased gradiently from 9.7 to 9.9 in the depth range of 0.5-2.5 cm. Within the depth range of 2.5-3.5, pH level was kept nearly stable at around 10.3, and when depth reached 3.5 cm, pH level went down to around 9.6 again.

Figure 1. Physicochemical profiles by microsensors



CARD-FISH and DAPI count statistics

According the physicochemical profiling properties, 6 layers of sediment were subjected to CARD-FISH probing and DAPI staining. The average probe and DAPI staining densities for each layer were listed in following tables (e.g. Slice 1A stands for the filter slice from Layer 1 subjected to probe A and DAPI.):

	Slices	*Probe counts*	*Area (μm^2)*	*Probe density (counts/area)*	*DAPI counts*	*Area (μm^2)*	*DAPI (counts/area)*	*average DAPI counts*	*abundance ratio*
Archaea	1A	74	5825.3492	0.0127	309	1397.8732	0.2211	0.2360	5.4%
Cytophaga/Flavobacteria	1B	78	5825.3492	0.0134	328	1234.8358	0.2656	-	5.7%
Cyanobacteria	1C	78	5825.3492	0.0134	299	1331.5155	0.2246	-	5.7%
Deltaproteobacteria	1D	112	5825.3492	0.0192	297	1469.718	0.2021	-	8.1%
Eubacteria	1E	-	-	-	-	-	-	-	-
Gammaproteobacteria	1F	70	5825.3492	0.0120	340	1275.4911	0.2666	-	5.1%
Nonsence	1G	-	-	-	-	-	-	-	-

	Slices	*Probe counts*	*Area (μm^2)*	*Probe density (counts/area)*	*DAPI counts*	*Area (μm^2)*	*DAPI (counts/area)*	*average DAPI counts*	*abundance ratio*
Archaea	2A	48	5825.3492	0.0082	245	1681.9936	0.1457	0.1888	4.4%
Cytophaga/Flavobacteria	2B	107	5825.3492	0.0184	220	1419.8814	0.1549	-	9.7%
Cyanobacteria	2C	60	5825.3492	0.0103	403	1783.8795	0.2259	-	5.5%
Deltaproteobacteria	2D	156	5825.3492	0.0268	326	1507.8102	0.2162	-	14.2%
Eubacteria	2E	-	-	-	-	-	-	-	-
Gammaproteobacteria	2F	35	5825.3492	0.0060	317	1576.4099	0.2011	-	3.2%
Nonsence	2G	-	-	-	-	-	-	-	-

	Slices	*Probe counts*	*Area (μm^2)*	*Probe density (counts/area)*	*DAPI counts*	*Area (μm^2)*	*DAPI (counts/area)*	*average DAPI counts*	*abundance ratio*
Archaea	3A	21	5825.3492	0.0036	188	1522.0114	0.1235	0.1610	2.2%
Cytophaga/Flavobacteria	3B	96	5825.3492	0.0165	252	1386.0263	0.1818	-	10.2%
Cyanobacteria	3C	39	5825.3492	0.0067	193	1279.8945	0.1508	-	4.2%
Deltaproteobacteria	3D	120	5825.3492	0.0206	230	1266.0897	0.1817	-	12.8%
Eubacteria (missed)	3E	-	-	-	-	-	-	-	-
Gammaproteobacteria	3F	49	5825.3492	0.0084	259	1549.956	0.1671	-	5.2%
Nonsence	3G	-	-	-	-	-	-	-	-

	Slices	Probe counts	Area (μm^2)	Probe density (counts/area)	DAPI counts	Area (μm^2)	DAPI (counts/area)	average DAPI counts	abundance ratio
Archaea	4A	46	5825.3492	0.0079	251	1466.964	0.1711	0.1656	4.6%
Cytophaga/Flavobacteria	4B	56	5825.3492	0.0096	231	1339.9344	0.1724	-	5.6%
Cyanobacteria	4C	11	5825.3492	0.0019	263	1416.1476	0.1857	-	1.1%
Deltaproteobacteria	4D	69	5825.3492	0.0118	235	1700.6712	0.1382	-	6.9%
Eubacteria	4E	-	-	-	-	-	-	-	-
Gammaproteobacteria	4F	40	5825.3492	0.0069	271	1689.7455	0.1604	-	4.0%
Nonsence	4G	-	-	-	-	-	-	-	-

	Slices	Probe counts	Area (μm^2)	Probe density (counts/area)	DAPI counts	Area (μm^2)	DAPI (counts/area)	average DAPI counts	abundance ratio
Archaea	5A	43	5825.3492	0.0074	210	1334.5655	0.1574	0.1772	4.2%
Cytophaga/Flavobacteria	5B	59	5825.3492	0.0101	228	1283.766	0.1776	-	5.7%
Cyanobacteria	5C	18	5825.3492	0.0031	242	1272.3435	0.1902	-	1.7%
Deltaproteobacteria	5D	55	5825.3492	0.0094	207	1267.266	0.1633	-	5.3%
Eubacteria	5E	-	-	-	-	-	-	-	-
Gammaproteobacteria	5F	16	5825.3492	0.0027	276	1398.1611	0.1974	-	1.6%
Nonsence	5G	-	-	-	-	-	-	-	-

	Slices	Probe counts	Area (μm^2)	Probe density (counts/area)	DAPI counts	Area (μm^2)	DAPI (counts/area)	average DAPI counts	abundance ratio
Archaea	6A	16	5825.3492	0.0027	167	1393.8717	0.1198	0.1427	1.9%
Cytophaga/Flavobacteria	6B	35	5825.3492	0.0060	197	1428.192	0.1379	-	4.2%
Cyanobacteria	6C	13	5825.3492	0.0022	251	1664.1768	0.1508	-	1.6%
Deltaproteobacteria	6D	50	5825.3492	0.0086	248	1604.1632	0.1546	-	6.0%
Eubacteria	6E	-	-	-	-	-	-	-	-
Gammaproteobacteria	6F	18	5825.3492	0.0031	188	1248.5681	0.1506	-	2.2%
Nonsence	6G	-	-	-	-	-	-	-	-

The abundance ratios of probe/DAPI counts (%) in layers were summarized and visualized in the Figure 2. Archaea abundance ratios were higher in the Layer 1, 2 and Layer 4, 5 with the value over than 4%. Bacteroidetes abundance ratios have their highest values in the Layer 2, 3 around 10%, so as to Deltaproteobacteria with the highest values in the Layer 2, 3 around 12-14%. Cyanobacteria abundance ratio profile has the general decreasing trend from the top layers to lower layers, ranging from around 6% to 1%. In the Layer 5, 6, Gammaproteobacteria abundance ratios have the lowest values along the profile around 2%.

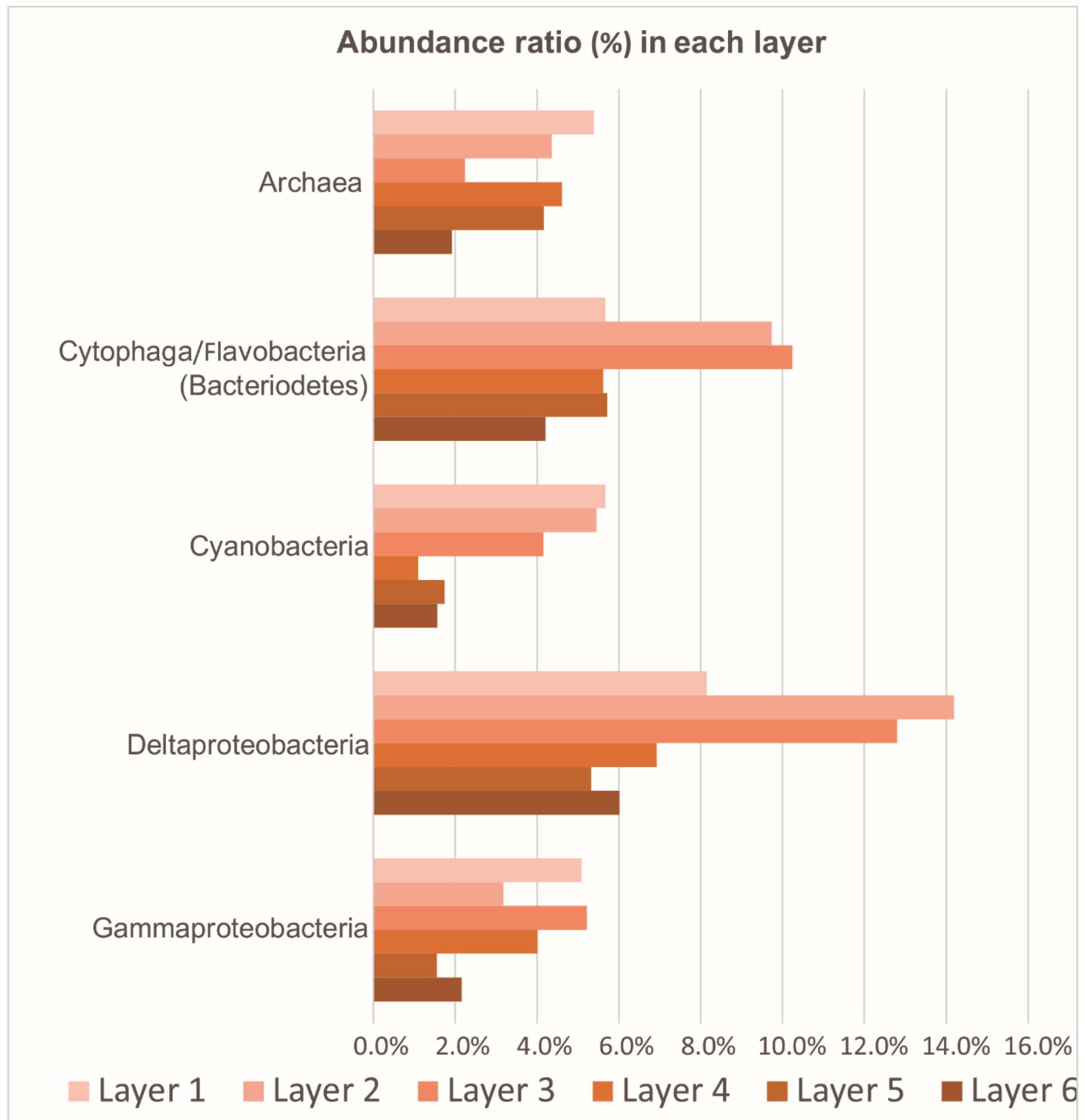


Figure 2. Abundance ratio of probe/DAPI counts in each layer

DAPI counts and qPCR results

The individual density of DAPI counts was applied to calculate back to the cell numbers on filter, and subsequently, the cell numbers/g wet sediment was obtained for each layer. From the following table, the cell numbers/g in all the layers are of the same magnitude, and a general deceasing trend appeared from the top layer to the lower layer, with the exception of layer 4.

Filter	*Depth (cm)*	*wet weight for CARD-FISH (g)*	*average DAPI counts (counts/area)*	*cell numbers/g*
1	0-0.5	0.441	0.2360	5.15E+08
2	0.5-1.0	0.364	0.1888	4.99E+08
3	1.75-2.25	0.316	0.1610	4.90E+08
4	2.75-3.25	0.3	0.1656	5.31E+08
5	3.75-4.25	0.382	0.1772	4.46E+08
6	4.75-5.25	0.354	0.1427	3.88E+08

In the following table, the cell numbers/g from each layer was back calculated by converting the 16S rRNA gene copies/g with the ratio of 3.6, which is the empirical average 16S rRNA gene copies per cell (Klappenbach et al. 2001). The cell numbers/g values from qPCR results are of the same magnitude with those by DAPI counting. Meanwhile, the top 3 layers also obtained high cell numbers/g than the lower 3 layers, which is also generally in accord with the decreasing trend of cell density along the profile reflected by DAPI counting.

Filter	*Depth (cm)*	*wet weight for DNA isolation (g)*	*16S rRNA gene quantities from qPCR (copies per µl)*	*16S rRNA gene (copies/g)*	*cell numbers/g (16S rRNA gene per cell ratio: 3.6:1)*
1	0-0.5	0.281	7.68E+06	2.73E+09	7.59E+08
2	0.5-1.0	0.266	8.60E+06	3.23E+09	8.98E+08
3	1.75-2.25	0.29	6.80E+06	2.34E+09	6.51E+08
4	2.75-3.25	0.312	4.45E+06	1.42E+09	3.96E+08
5	3.75-4.25	0.254	4.26E+06	1.68E+09	4.66E+08
6	4.75-5.25	0.317	6.20E+06	1.95E+09	5.43E+08

Discussions

Transitionary microbial community

For archaea, there will be some aerobic archaeal groups, such as Thaumarchaeota, present. Thaumarchaeota FSCG will be responsible for autotrophic ammonium oxidization, and that the same time, there is also the mixotrophic evidence for Thaumarchaeota, such as *Ca*. Nitrososphaera gargensis containing the transporters for various organic matters in its genome inventory (Spang et al. 2012). This group of aerobic archaea group might be the major components in the top layers. In the anoxic lower layer, some other microbial groups, such as Bathyarchaeota and MBG-B might be the major components, for both of them living an anaerobic and heterotrophic lifestyle based on the buried organic carbons (Lloyd et al. 2013; Teske and Sørensen 2008). Cyanobacteria will need light to perform the photosynthesis, and the drastic decreasing trend of their abundance in depth profile is reasonable for these traits (Whitton and Potts 2012).

The Cytophaga and Flavobacteria are mainly being characterized as aerobic, capable for utilizing macromolecules, such as proteins and polysaccharides and widely distributed in aquatic and terrestrial environments (Rosenberg et al. 2014b). Their more abundant distribution in upper layers will be attributed to these traits. For Gammaproteobacteria, it was decreasing along the depth profile as a whole, probably due to large parts of its components could be aerobic (Rosenberg et al. 2014a). And meanwhile, Gammaproteobacteria is a huge branch that it will be not wise to make any conclusive points on this distribution associated with eco-chemicals. However, PSB, which could grow best at the moderate low sulfide concentration and depends on the light availability, is one of the components within Gammaproteobacteria. The more abundant distribution in the top layer of Gammaproteobacteria could be more or less related to that the conditions in top layer with lower sulfide conc. and high light availability could benefit the growth of PSB.

Deltaproteobacteria is the most abundant group within all layers. And many of its components could be the sulfate or sulfur reducer groups, such as Syntrophobacterales, Desulfobacterales, Desulfobacterales and Desulfarculales; and Desulfuromonas. Most of them could chemoorganoheterotrophically degrade and obtain energy from large organic molecules and subsequently acquire incomplete oxidized products as acetate or complete oxidized products as carbon dioxide (Rosenberg et al. 2014b). However, unfortunately, there were not corresponding physicochemical parameters to explain this. Its distribution is co-abundant with the distribution of H_2S concentration. Could it be the results of the active consequence of sulfate reducing of Deltaproteobacteria? More evidence and details should be provided to make the conclusion like this.

DAPI and qPCR

This study provides good accordance evidence on the two microbial quantification method. From DAPI, a more intuitive result could be obtained under the microscope, and will be more reliable for quantification. For qPCR, taking 16S rRNA gene copy number as the proxy to back calculate cell numbers could also be feasible and reliable. Considering the time-consuming and labor issue, qPCR will be more practical for most of the cases and will provide as good results as direct DAPI counting.

This miniproject still could not address the accurate microbial distribution pattern of “rainbow soil”, however, the quantitative CARD-FISH and DAPI counting method could still reflect the changes of major microbial groups in depth profile. And these discoveries provided in this study will facilitate the decision of choosing sediment types for anoxygenic phototrophs enrichment. It still reflected the general physicochemical profile distributing pattern in the shore sediment of Trunk River. If there is still some more time left, I will use one more CARD-FISH probe for Chlorobi, for detecting the green sulfur bacteria. And in order to get more quantitative distribution pattern information of integral microbial groups, I think MiSeq based 16S rRNA gene profiling will be the good choice to achieve that.

Protocols and Details

(mainly according to Work Manual of MicDiv Year 2016. Some key parameters and order were changed to get good performance.)

Microsensoring

I *O_2 microsensor*

The O_2 sensor is an amperometric Clark-type electrode. The electrode is pre-polarized before use and oxygen from the environment diffuses across a permeable membrane at the tip and reduces at the cathode. Two-point calibration was applied. To calibrate the O_2 sensor for 100% [O_2] value, one should bubble a salt solution with the same salinity level as your sample (0.45% in this study) with atmospheric oxygen for 20 min, and heat it at the same time to allow it to reach the same temperature as your sample. To calibrate the O_2 sensor for 0% [O_2] value, one will use the anoxic solution instead. After measurements, to clean the oxygen electrode, rinse in 95% EtOH, followed by 0.01 M HCl, and finally DI H_2O.

II *H_2S microsensor*

The H_2S microelectrode measures the signal of the re-oxidation of ferrocyanide at its anode tip that is generated from HS^- molecules from the diffusion of H_2S driven by partial pressure into an alkaline electrolyte solution. The HS^- initially reacts with ferrocyanide producing sulfur and ferrocyanide. Similar to the oxygen sensor, the reduction current is converted to voltage signal. Calibrate the electrode by DI water (0) and Na_2S solution (100 μM, in anaerobic tube with N_2 headspace). After measuring, rinse the electrode in 70% EtOH, followed by DI H_2O.

III *Redox microsensor*

The redox working electrode develops an electrical potential relative to the reference electrode and is an indication of electron flow. The redox electrode is a platinum rod connected to a reference electrode. Two quinhydrone redox buffer solutions are required: 1) Mixing 50 ml of pH 4 buffer solution with 0.5 g of quinhydrone, 2) Mixing 50 ml of pH 7 buffer solution with 0.5 g of quinhydrone. Crystals of quinhydrone should be present to indicate that the solution is saturated. Heat both of tow buffer to the same temperature as your sample. One could place the electrode into the cooling buffer solution to equilibrate for long time. Under 37°C, the potential of pH 4 and 7 buffer should be 442.8 and 268, respectively; and one should recalculate the values for other

temperature. Redox electrodes (working and reference electrodes) were calibrated by placing two electrodes into each buffer and using the potential difference of two buffer solutions to calculate. After measurements, wipe the outside of the Ref-N with Kim-wipe, rinse in 70% EtOH, followed by DI H_2O. The Ref-N should be stored in 3M KCl.

IV *pH microsensor*

pH electrode is a potentiometric sensor which develops a signal based on the diffusion of protons and difference in the potentials between the pH electrolyte and a reference. pH glass is very weak and the easiest to break of all the sensors. Place both of the working and reference electrodes into at least three different pH buffers (e.g. 4, 7, 10). After measurement, clean the Ref-N by wiping the outside with Kimwipe and rinse the electrode in DI H_2O. Rinse the working electrode in 70% EtOH or 0.01M HCl for a few minutes. After, rinse with pH 4 buffer. Store the working electrode in DI H_2O and the Ref-N in 3M KCl.

The automatic profiling and data acquisition by software SensorTrace Pro 2.3. The vertical intervals were 2000 μm and vertical profile were taken in 7 cm depth for O_2, H_2S, and redox potential and in 4 cm depth for pH value. At each measuring point, 3 seconds was used, and between two measuring point, 1 second was used. Four parameter profiles were acquired by swapping electrode after the other, respectively. Data were exported according to the manual.

CARD-FISH

I *Fixation*

1. Fix sediment samples with fresh paraformaldehyde (PFA) solution (0.3~0.4 g wet sediment samples with 700μl PFA, final concentration 4%) over night at 4°C. Vortex to well mix samples and PFA solution before fixation.
2. Centrifuge at 16000 × *g* for 5 min; discard supernatant (in the container for toxic PFA waste) and resuspend sample with 1×PBS (pH 7.6).
3. Repeat step 2 twice.

II *Sonication*

1. Put fixed sediment sample on ice and sonicate it five times for 30 sec with a 30 sec break in between with 1 pulse per second and an intensity of 20% (BRANSON sonifier 250).
2. Filtrate all the supernatant onto polycarbonate filter (0.2 μm, GTTP, Merck Millipore) at a pressure of around of -200 mbar.
3. Dry the filter in oven at 46°C. Store the filters in -20°C refrigerator.

III *Embedding*

1. Boil low melting agarose (0.1%, gel strength should be approx., 1000 g cm^{-2}).
2. Let it cool down to 35-40°C.

3. Dip filter with both sides in the agarose and place it face-up onto a parafilm covered, even surface (e.g. glass plate) or a Petri Dish.
4. Dry the filter in oven at 46°C.

IV *Inactivation of endogenous peroxidases*

1. Incubate in H_2O_2 (0.15% in methanol) for 30 min at RT (15ml is enough for 6 filters incubated in one petri dish).
2. Wahs filters well in excess MilliQ water.
3. Wash 96% ethanol and dry the filter in oven at 46°C.
   (after this step, the filters could be stored in -20°C freezer for long time (half or one year). Cut filter into slices, the rest slices are left stored further probe testing.)

V *Permeabilization*

Using lysozyme (optimal method for most of the pelagic and benthic bacteria)

1. Incubate filter in fresh lysozyme solution (10 mg ml^{-1} lysozyme (100 kU mg^{-1} from chicken egg white, SIGMA) in 0.05M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0) for 45 min at 37°C.
2. Wash in excess MilliQ water and wash 96% ethanol and dry the filter in oven at 46°C.
   (after this step, the filters could be stored in -20°C freezer for overnight.)

Using proteinase K (optimal method for most of the pelagic and benthic archaea)

1. Incubate filter in fresh Proteinase K solution (20 μg ml^{-1} Proteinase K (2.5U mg^{-1} from *Tritirachium album*, as solution by Macherey-Nagel) in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl) for 10 min at RT.
2. Wash in excess MilliQ water and wash 96% ethanol and dry the filter in oven at 46°C.

VI *Hybridization*

1. Mix hybridization buffer with probe working solution (50 ng DNA μl^{-1}) in a ratio 300:1
2. Prepare 500 μl hybridization solution in a 2ml vial (Ep tube) and place the filter sections (facing inwards) in the vial (500 μl is enough for at least 6-8 filter sections).
3. Incubate at 46°C for 2-3 hrs.

VII *Washing*

1. Transfer filters to the washing buffer using tweezers.
2. Washing filters in pre-warmed washing buffer (15 min, 48°C).
3. Transfer filters to 1×PBS (do not let filter run dry!) and incubate for 10 min at RT.
4. To remove excess liquid, dab filter on blotting paper, but do not let filter run dry.

VIII *CARD*

1. Prepare a fresh solution of H_2O_2 (0.15% in PBS), keep it cool.
2. Mix amplification buffer with 0.15% H_2O_2 solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient).
3. Add fluorescently labeled tyramide (1 mg ml^{-1}) and mix well, keep in the dark (The volume of labeled tyramide added, strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: increase the ratio of added tyramide.).
4. Dip filter completely in the amplification mix in a 2 ml vial and incubate at 46°C for 30 min in the dark.
5. Dab filter on blotting paper and incubate in 1×PBS for 10 min at RT in the dark.
6. Wash filters thoroughly in excess MQ and 96% ethanol (~1 min), let completely air dry in the dark before counterstaining with DAPI.
   DAPI
   [1. Add DAPI working solution ~20 µl on the petri dish, dip the face side of slice on each droplet. Let it stand for 5 min.
   2. Wash filters in water and ethanol.
   The stocking solution of DAPI concentration is 1mg/ml (dissolved in water)]
7. To keep the background fluorescence low, wash filters in large volume of water and ethanol.
8. Add mounting medium to make the slides and store the slides in -20°C freezer without apparent loss in CARD-FISH signal intensity (lasting for 1 year).

The protocols for making hybridization and washing buffer, and CARD amplification buffer were listed in Appendix.

Appendix

Buffers for CARD-FISH

MATERIAL

- Metaphor (low melting) agarose
- 10% Blocking Reagent (Roche, Basel, Switzerland)
- Dextran sulfate
- Enzymes (Lysozyme and/or Proteinase K)
- HRP-labeled oligonucleotide probes [50 ng μL^{-1}] (Table 5, store at 4°C, do not freeze!)
- Working solution with dye-linked tyramides (Fluorescein, Cy3, Cy5 or Alexa dyes)
- 4-iodophenylboronic acid (IPBA, only needed to prepare tyramide working solution)

STOCK SOLUTIONS

- 0.01 M HCl solution
- Methanol
- 30% hydrogen peroxide solution (Attention: H_2O_2 is a very strong bleach and oxidant, protect your skin and eyes!)
- Blocking solution (10% blocking reagent in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) prepared according to manufacturer's instructions)
- CARD hybridization buffer (see below)
- CARD amplification buffer (see below)

CARD hybridization buffer
(final volume 20 ml)

- 3.6 mL 5 M NaCl
- 0.4 mL 1 M Tris HCl, pH 8.0
- 20 µL SDS (20% w/v)
- 2.0 mL blocking solution
- X mL formamide (depending on oligonucleotide probe, see Table 1 and Table 5)
- X mL sterile milliQ H_2O (depending on the formamide concentration that is needed for the oligonucleotide probe, see Table 1 and Table 5)
- 2.0 g of dextran sulfate
- Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. Small portions of the buffer can then be stored at –20°C for several months.

Attention: Formamide is toxic, please protect yourself and process all steps involving formamide in the fume hood!

Note: The formamide concentration is dependent on the probe used and determines the stringency of the hybridization. Hybridization stringency may also be adjusted by temperature rather than by the chemical composition of buffers. We find that it is more convenient to keep incubator and water bath at one set temperature and to modulate the stringency by adding formamide.

Washing buffer

(produce freshly when needed, final volume 50 ml, see also Table 2)

- 0.5 mL 0.5 M EDTA, pH 8.0
- 1.0 mL 1 M Tris HCl, pH 8.0
- X µL NaCl (depending on formamide concentration in the hybridization buffer, see Table 3)
- add sterile milliQ H_2O to a final volume of 50 mL
- 25 µL SDS (20% w/v), add SDS last to prevent precipitation

The NaCl concentration in the washing buffer, as well as the formamide concentration of the hybridization buffer determines the stringency of the hybridization at the selected temperature.

CARD amplification buffer

(final volume 40 ml)

- 4 mL of 10 × PBS, pH 7.4 (important for proper enzyme function)
- 0.4 mL blocking solution
- 16 mL 5 M NaCl
- Add sterile milliQ H_2O to a final volume of 40 mL
- Add 4 g of dextran sulfate

Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. The amplification buffer can be stored in the refrigerator for several weeks.

Table 1: Volumes of formamide and water for 20 mL of hybridization buffer

% formamide in h-buffer	mL formamide	mL water
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

Table 2: Standard washing buffer

Stock reagent	Volume	final concentration in washing buffer
5 M NaCl		concentration depending on % formamide in hybridization buffer (see Table 3)
1 M Tris / HCl	1 mL	20 mM
0.5 M EDTA*	500 µL	5 mM
20% SDS**	25 µL	0.01%
milliQ H_2O	add to 50 mL	

*only if 20% formamide or more!

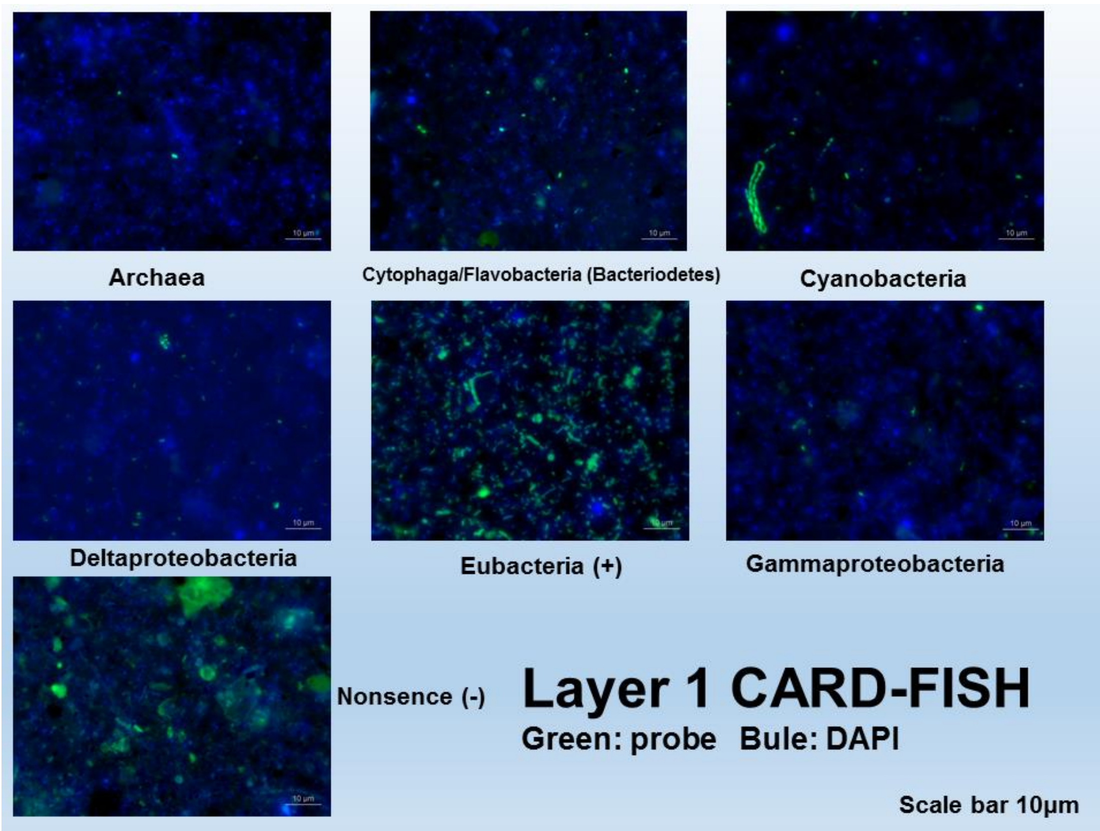
** add SDS last to avoid precipitation

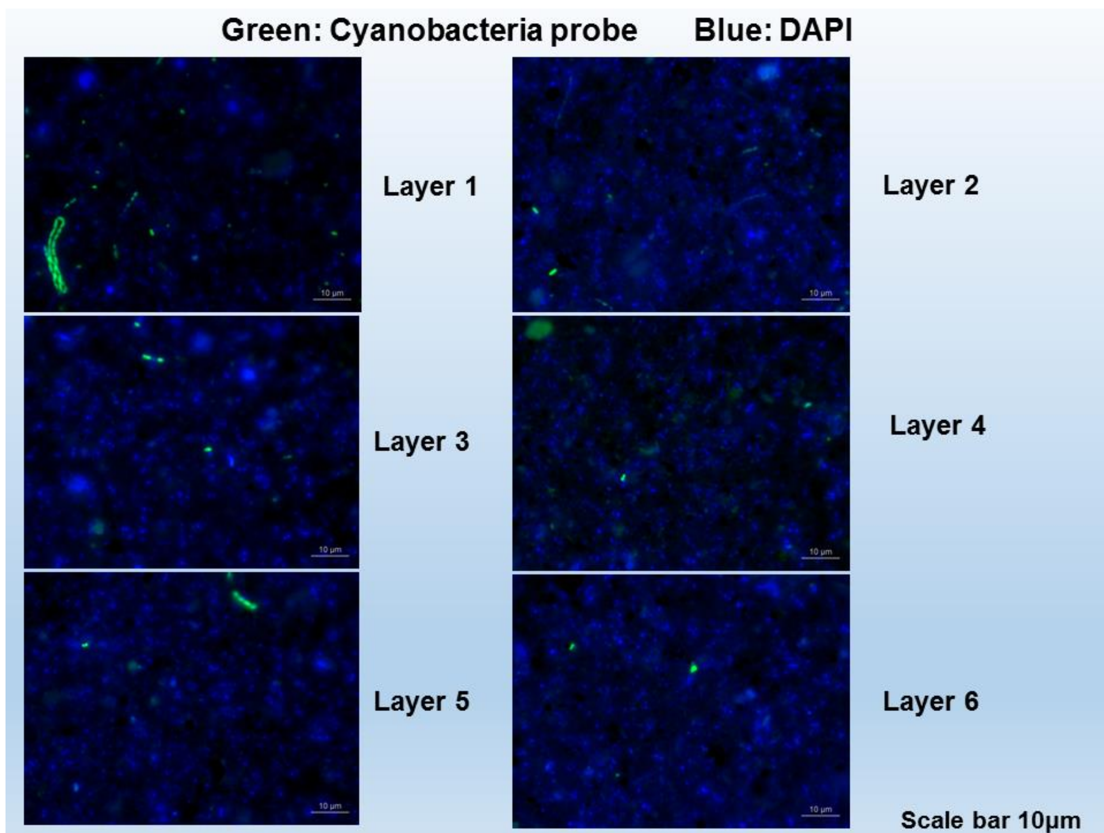
Table 3: Corresponding NaCl concentration in 50 mL washing buffer at 48°C

% formamide in hybridization buffer	[NaCl] in Mol	µL 5 M NaCl
0	0.900	8900
5	0.636	6260
10	0.450	4400
15	0.318	3080
20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	-	-

Note: The stringency in the washing buffer is achieved by adjusting the NaCl concentration, which is calculated using the formula from Lathe et al. The addition of EDTA contributes to the Na^{+} concentration, therefore the required volume of 5 M NaCl solution in the washing buffer is reduced by 100 µL.

CARD-FISH images





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