

Characterization of deep-sea microbial activities in a whale-fall sediment

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Abstract

In the deep-sea sediment, microbes can form a unique community when a large amount of nutrient load is spiked, for example, as a result of the introduction of a whale-fall. In this project, a sediment core from a whale-fall site at 1820 m depth in Monterey Canyon, California was used for studying whale-fall sediment microbial community and their activities. Measurement of methane concentration in whale-fall sediment microcosms by gas chromatograph (GC) showed that treatments with different substrates for anaerobic respiration, including methanol, hydrogen gas, acetate, and propionate, could promote methanogenesis within a week after the initiation of the microcosm incubation. Simultaneous treatment with sulfate did not have significant effects on the methane production. Compared to methanol and hydrogen gas, effects of acetate and propionate were smaller. Uptake of acetate and propionate in these microcosms were observed by high pressure liquid chromatography (HPLC) analyses, indicating that these substrates are promoting methane production. Sequencing and phylogenetic analyses of archeal 16S rDNA and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis of *mcrA*, encoding Methyl coenzyme M reductase α -subunit (*mcrA*), indicated the presence of methanogenic archaea in the whale-fall sediment. T-RFLP analysis of bacterial 16S rDNA gene from whale-fall and control sediment sample indicated that the bacterial community compositions in two samples are different. Overall, the results indicated that methanogenesis can be the prevalent terminal metabolism in the whale-fall sediment environments, which harbor unique microbial communities enriched with methane-producing archaea.

Introduction

In anaerobic conditions, sulfate reduction by sulfate reducing bacteria (SRB) is generally thought to preclude methanogenesis for thermodynamic reasons. As SRB compete for substrates that are also utilized by methanogens, methanogenesis is usually outcompeted by sulfate reduction and is restricted to a sulfate-depleted zone in marine sediments. While such spatial separation is generally thought to facilitate methane production in

marine sediments, in some unique environmental conditions, methanogenic archaea are found to coexist with SRB. One such environment is the whale-fall sediment. A whale-fall is a whale carcass, which has fallen to the deep-sea sediment. A measurement conducted by Goffredi et al. (2008) showed that the concentration of sulfate in whale-fall sediment at 0-1 m depth can range from approximately 10 to 30 mM (Goffredi et al. 2008). In spite of the presence of sulfate that can promote the activities of SRB, the study also showed that methanogenic archaea can represent up to 40% of the cell counts in the system.

The major objective of this project was to explore conditions that promote methane production in a sediment sample collected by a whale-fall site, and to characterize archaeal community structure using molecular approaches. Activities and community structure of methanogenic archaea were explored by using multiple different approaches, including microcosm and molecular analyses (Figure 1). To assess changes in methanogenic activities in response to treatments with different substrates for anaerobic respiration, microcosms with sediment samples collected in a whale-fall site were incubated with acetate, propionate, butyrate, methanol, and hydrogen gas with or without sulfate. Activity measurements indicated that methanogenesis in the sediment can be promoted within a couple of days after the initiation of the incubation, and microbial responses can vary depending on the substrate amended to the microcosms. Molecular analyses of 16S rDNA diversity suggested that methanogenic archaea are relatively abundant in the community. Furthermore, comparisons of whale-fall and control sediment samples by T-RFLP indicated that bacterial community in the whale-fall site can also be different from that of the control sediment.

Methods

A. Setting up microcosms and incubation conditions

A 0-9 cm sediment core sample from a whale-fall site at 1820 m depth in Monterey Canyon, California was used for microcosm experiments. The whale-fall, Prtrick, have been deposited on the sea-floor for approximately two years before the sediment sampling for this project. The sediment sample used for this project was stored at 4 °C

until the experiment. The control sediment sample was obtained by Dr. Collen Cavanaugh's group near the Naushon Island, MA.

A modified basal anaerobic modular medium was used for slurry preparation. To a round-bottom flask, 1 L of 1x SWB (seawater base), 20 ml of 1M MOPS, pH 7.2, 1 ml of 1 M K_2HPO_4 , 1 ml of 1000 x trace element solution, and 1 ml of 1000 x vitamine solution were added. The medium was boiled for 10 minutes and cooled under a flow of anaerobic N_2/CO_2 (80%/ 20%). The flask was moved to an anaerobic chamber and 70 ml of 1 M $NaHCO_3$, 10 ml of 0.5 M NH_4Cl , 2 ml of cysteine -HCl. 2 ml of 0.2 M Na_2SO_4 was also added right before setting up the microcosms. The 1 L medium was divided into two flasks and 14 ml of 1 M $NaSO_4$ was added only to one of the flasks (Table 1). 150 g of sediment was added to each flask and mixed well with the medium. 20 ml of the slurry was aliquoted into each serum bottle used for microcosm incubation. 60 ml serum bottles were used. Each microcosm was treated with 2mM acetate, 2mM propionate, 2mM butyrate, 50ul of 100% methanol, or high hydrogen concentration in the headspace (Table 1). The microcosms were incubated at 4 °C for 18 days.

B. HPLC analysis to monitor organic acid uptake in microcosms

LC-2010CHT liquid chromatograph (Shimazu Scientific Instruments, Inc, Nowell, MA) was used to monitor organic acid uptake in microcosms. From each microcosm, approximately 1 ml of slurry was sampled using a syringe and transferred to a 1.5 ml tube. The slurry was centrifuged at maximum speed for 5 min and 600 ul of supernatant was transferred to HPLC vials. HPLC analyses were performed five times over the course of this project. EZStart 7.4 was used for identifying acetate, propionate, and butyrate peaks.

C. GC analysis to monitor methane production in microcosms

GC2014 Shimazu Gas Chromatograph (Shimazu Scientific Instruments, Inc, Nowell, MA) was used to monitor methane production in microcosms. For each microcosm, 100 ul of headspace air was sampled. GC analyses were performed five times over the course of this experiment. EZStart 7.4 was used for identifying methane peak.

D. Cline assay to monitor sulfide production in microcosms

Sulfide (Cline) assay was performed to monitor sulfide production in microcosms. To each 50 ml conical tube, 25 ml of MQ water and 1 ml of 20 % (w/v) zinc acetate (water was used as a solvent) was added. Then, 0.3 ml of liquid from each microcosm was sampled using a syringe, and mixed with the zinc acetate solution. The liquid sample was not exposed to the air. To each conical tube, 0.5 ml of PDA/ferric reagent (6 g of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) mixed with cooled 50% (vol/vol) hydrochloric acid) was added and mixed well. 500ul of the solution from each sample was transferred to a cuvette and absorbance at 670 nm was measured by using a spectrophotometer.

E. Archeal 16S rDNA phylogenetic analysis

DNA was extracted from whale-fall and control sediment samples used for microcosms by using soil DNA isolation kit (MO BIO Laboratories, Inc.). Primers, 1AF and 1100R were used. Polymerase chain reaction (PCR) condition used in this experiment included 3-minute denaturation at 95 °C, 26 cycles of 30-second denaturation at 95 °C, 30-second annealing at 55 °C, and 75-second elongation at 72 °C, followed by 3-minute elongation at 72 °C. PCR amplicons were cloned using the Topo TA cloning kit (Invitrogen). 96 clones were selected and sequenced at the Keck Facility at the MBL Josephine Bay Paul Center. Sequence alignment and phylogenetic tree construction were performed by using Arb (<http://www.arb-home.de/>).

F. T-RFLP analysis of bacterial 16S rDNA, *AprA*, and *McrA* diversity profiles

T-RFLP analyses were performed to assess bacterial 16S rDNA, *AprA*, and *McrA* diversity in whale-fall and control sediment samples used for the microcosm experiments. The same DNA extracted for the archeal 16S rDNA phylogenetic analysis was used to PCR amplify all three genes.

For analysis of bacterial 16S rDNA diversity profile, primers, 8F labeled with Hex dye and 1492R, were used. PCR condition used in this experiment included 3-minute denaturation at 95 °C, 30 cycles of 30-second denaturation at 95 °C, 30-second annealing at 46 °C, and 30-second elongation at 72 °C, followed by 3-minute elongation at 72 °C. PCR product was purified using QuickStep™ 2 PCR purification kit (Edge

Bio). Two separate restriction enzyme digestions of purified PCR product were performed with *Sau96 I* and *Rsa I* (New England Biolabs). 300 ng of DNA was used for each reaction.

For analysis of bacterial *AprA* sequence diversity profile, primers, *AprA*-1-FW (5'-TGGCAGATCATGATY MAYGG-3') labeled with Hex dye and *AprA*-10-RV (5'-CKGWAGTAGWARCCRGGRTA-3'). A combination of two gradient cycles was used for PCR amplification of the gene. Followed by 5-minute denaturation at 95 °C, 20 cycles of 45-second denaturation at 95 °C, 60-second annealing at 50-59 °C, and 90-second elongation at 72 °C were performed. Then, 14 cycles of 45-second denaturation at 95 °C, 60-second annealing at 50-54 °C, and 90-second elongation at 72 °C was performed, followed by a 5-minute elongation at 72 °C. PCR product was purified using QuickStep™ 2 PCR purification kit, and 300 ng of the purified PCR product was digested with *Rsa I*.

For analysis of archeal *mcrA* sequence diversity profile, primers, *mcrA*-f labeled with D2 dye (5'-GGTGGTGT MGGATTCACACAR-3') and *mcrA*-r (5'-TTCATTGCRTAGTTWGGRTAG-3') (Luton et al. 2002) were used. PCR condition used in this experiment included 3-minute denaturation at 95 °C, 40 cycles of 60-second denaturation at 95 °C, 60-second annealing at 50 °C, and 60-second elongation at 72 °C, followed by 6-minute elongation at 72 °C. PCR product was purified using QuickStep™ 2 PCR purification kit, and 300 ng of the purified PCR product was digested with *Rsa I*.

All samples were subsequently sequenced at the Keck Facility at the MBL Josephine Bay Paul Center. Different T-RFLP profiles were compared using GeneMarker V1.85 (<http://www.softgenetics.com/>).

Results and Discussion

A. HPLC analysis to monitor organic acid uptake in microcosms

Organic acid uptake by microbes in the whale-fall sediment was monitored by using HPLC. Acetate, propionate, and butyrate are intermediate products produced during anaerobic respiration, and propionate and butyrate have to be oxidized to acetate before being utilized by methanogens. Overall, the analysis showed that microbial communities first utilized the most favorable substrate, acetate (Figure 2A). Once acetate became

unavailable, the community started consuming propionate (Figure 2B) and then butyrate (Figure 2C).

While 2 mM of each organic acid was added to the slurry with or without sulfate, the initial HPLC analysis showed that the concentration of the substrates added to the microcosms were approximately 1.5 mM. Nine days after initiation of the microcosm experiment (July 8th), the levels of acetate in all four microcosms amended with the substrate (Table 1) became 0 mM, indicating that the microbes in the whale-sediment have used up the substrate completely (Figure 2A). Fifteen days after initiation of the microcosm experiment (July 22nd), the levels of propionate in all four microcosms amended with the substrate (Table 1) became 0 mM. Eighteen days after initiation of the microcosm experiment (July 25th), the levels of butyrate in the two microcosms amended with butyrate without sulfate have decreased to 0 mM. The levels of butyrate in the other two bottles treated with sulfate were 17 % (microcosm 9) and 16 % (microcosm 10) of original levels. The levels of acetate, propionate, or butyrate in microcosms with other treatments, including controls, were not detectable. No obvious difference was observed between microcosms with and without sulfate treatment.

B. GC analysis to monitor methane production in microcosms

Methane production in microcosms was monitored over time to investigate how methanogens in whale-fall sediment microcosms respond to treatments with different substrates for anaerobic respiration. Five measurements were performed over the course of 2.5 week experiment. The substrates for methanogenesis, including acetate, propionate, methanol, and H₂ gas had impacts on methane production even when the treatments were combined with sulfate amendment. The results showed that methanol and high concentration of hydrogen gas have promoted methanogenesis more than other treatments (Figure 3A). As SRB do not compete for methanol, the response of the methanogenic community to the methanol treatment selectively facilitated the activity of methanogenic archaea. In contrast to methanol, both SRB and methanogenic communities compete for H₂ gas. Interestingly, the effect of hydrogen gas treatment on methane production was still observed in the whale-fall sediment microcosms. The impacts of

methanol and hydrogen gas treatment also seemed larger and more immediate compared to those by other treatments.

The community also responded to the treatments with organic acids but the level of methane concentration was lower compared to those treated with methanol and H₂ gas. In figure 3B, only the levels of methane concentration in microcosms treated with acetate, propionate, and butyrate were compared to those in control microcosms (microcosm 21-28). The analysis focusing on the samples treated with organic acids showed that the levels of methanogenesis increased first within the microcosms treated with acetate. The initial increase was observed between six (July 13th) and nine (July 16nd) days after initiation of the microcosm experiment. There was no obvious difference between acetate-treated microcosms with and without sulfate.

Next, increase in methane production was observed in microcosms treated with propionate (Figure 3B). Microcosms treated without sulfate started showing elevated methane concentrations in the headspace between six (July 13th) and nine (July 16nd) days after the initiation of the microcosm experiment. While the stimulation of methanogenesis in microcosms treated with propionate without sulfate occurred almost simultaneously as that in microcosms treated with acetate, the extent of the increase in methane concentration was less than those of acetate-treated microcosms. The levels of methane concentration in propionate-treated microcosms without sulfate amendment have started increasing between 9th (July 16th) and 15th (July 22nd) days after the initiation of the experiment. The methane concentration in propionate-treated microcosms without sulfate continued to be higher than those with sulfate even 18 days of incubation (July 25th).

The levels of methane in microcosms treated with butyrate did not start increasing even after 18th days of incubation (the last measurement performed in this project). Interestingly, the HPLC analysis of these microcosms showed that butyrate has been utilized by organisms in the sediment samples. The result indicated that the conversion of butyrate to acetate would require more than 18 days, and therefore, utilization of the substrate by methanogenic communities would start in a later time point.

The changes in the levels of methane production in response to hydrogen gas treatment was compared in the whale-fall and control sediment microcosms.

Interestingly, the level of methane production was only observed in the whale-fall sediment microcosms (Figure 4).

C. Cline assay to monitor sulfide production in microcosms

The cline assay for monitoring sulfide production by sulfate reducers did not detect any sulfide in microcosms even in the whale-fall sediment. However, the assay did not detect sulfide even in control sediment, which had strong odor of sulfur. (When the whale-fall sediment microcosms were opened in the end of the microcosm experiment, the sediment did not have the odor of sulfur.)

D. Archeal 16S rDNA phylogenetic analysis

Phylogenetic analysis of archeal 16S rDNA in whale-fall sediment used in this project showed that many phlotypes in this sediment were very similar (or almost identical) to sequences identified in a previous whale-fall study (Goffredi et al. 2008) (Figure 5). The analysis also showed that the many sequences identified in this sample were almost identical one another, suggesting that the archeal community in this whale-fall sediment is dominated by a few major groups. Methanosarcina and Methanogenium within the phylum Euryarchaeota as well as Thermofilaceae within the phylum Crenarchaeota were the most abundant groups abundant within the whale-fall sediment sample.

Methanosarcina and Methanogenium were also found to be abundant methanogenic group in a previous study conducted by Goffredi et al. (2008). Less abundant phlotypes identified in this whale-fall sediment including, Methanococoides within the phylum Euryarchaeota and Thermoplasma within the phylum Crenarchaeota, were also found in the study conducted by Goffredi et al. (2008). The analysis also revealed the presence of a cluster of unclassified Euryarchaeota phlotypes. The closest relatives of these phlotypes in the Silva database (<http://www.arb-silva.de/>) was found to be phlotypes previously identified in the hydrothermal vent environment (Takai and Horikoshi 1999).

E. T-RFLP analysis of bacterial 16S rDNA, *AprA*, and *McrA* diversity profiles

A community finger printing analysis using T-RFLP suggested that the community composition of bacteria in the whale-fall and control sediments are different each other

(Figure 6 A and B). The result suggests that the whale-fall sediment harbors a unique community of microbes that can potentially behave very differently compared to the deep-sea sediments that are not experiencing sudden increases in nutrient levels.

Dissimilatory adenosine-59-phosphosulfate (APS) reductase (AprBA) is a key enzyme required for the dissimilatory sulfate reduction by bacteria (Meyer and Kuever 2007). In this project, primers targeting α subunit of the enzyme, AprA, have been used (Meyer and Kuever 2007). T-RFLP analyses of *AprA* diversity profiles in the whale-fall and control sediments showed that sulfate reducing bacteria that are present in both sediments can be similar each other (Figure 7).

Methyl coenzyme M reductase α -subunit (*mcrA*) is an enzyme expressed by methanogenic archaea and has been broadly utilized to assess diversity of methanogens in the environment (Luton et al. 2002). Primers developed by Luton et al. (2002) were used for assessing diversity profile of *McrA* gene in the whale-fall sediment. T-RFLP analyses of *McrA* diversity profile was only performed for the whale-fall sediment as amplification of the gene has failed for the control sediment (Figure 8). It is possible that the level of methanogens within the control sediment community was low and therefore could not be amplified by PCR. While it is possible that the biases associated with the PCR amplification condition and/or primer target positions prevented amplifications of the gene in the control sediment, the negative result obtained in this PCR gives interesting implications. First, other genes, including 16S rDNA of bacteria as well as *aprA* were successfully amplified from the same genomic DNA template (Figure 6 and 7), suggesting that the amplification failure was probably not due to the problems associated with DNA template. Second, the primers were also successfully used by Harrison et al (2009) and other groups (Luton et al. 2002). Finally, the methane production in the control sediment was not observed in GC analysis discussed earlier, suggesting that the level of methanogenic activities are very low in the control sediment.

Future direction

FISH and DAPI counts of microbial cells in the whale-fall sediment before and after incubation with substrates have not been conducted because of the limitation. However, sediment samples fixed with 2% paraformaldehyde is currently stored at -20 °C, and the

analyses can potentially be conducted in the future. Likewise, sediment samples from different time points during microcosm incubation have been stored at -80 °C for future molecular analysis. Changes in community compositions by using T-RFLP primers against bacterial 16S rDNA, *aprA*, *mcrA* can be conducted in the future.

Acknowledgement

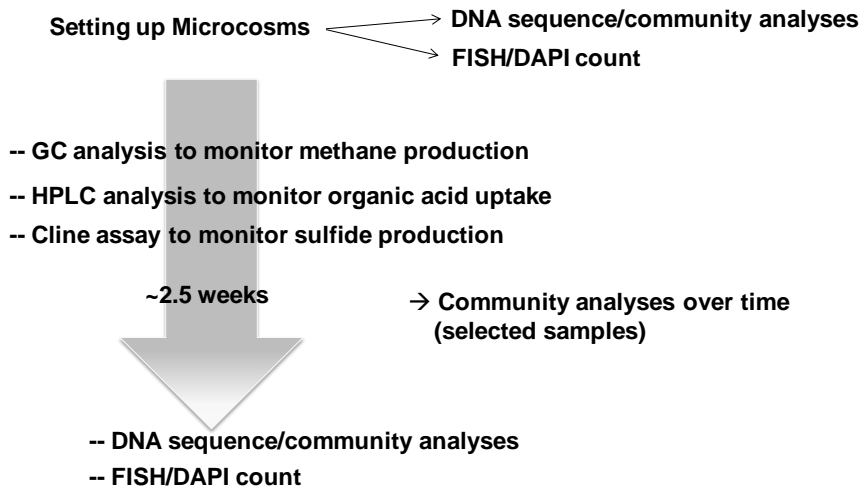
I would like to thank Dr. Victoria Orphan for allowing me to use her precious sediment sample from the whale-fall site, giving me many great suggestions, and helping me with interpretation of the data. I also would like to thank Alexa Price-Whelan for her help with media preparation, HPLC, and GC analyses. I also would like to thank Hinsby Cadillo-Quiroz for his assistance with all the community analysis using molecular approaches. I also would like to thank Dr. Stephen Zinder for valuable suggestions and assistance with GC operation.

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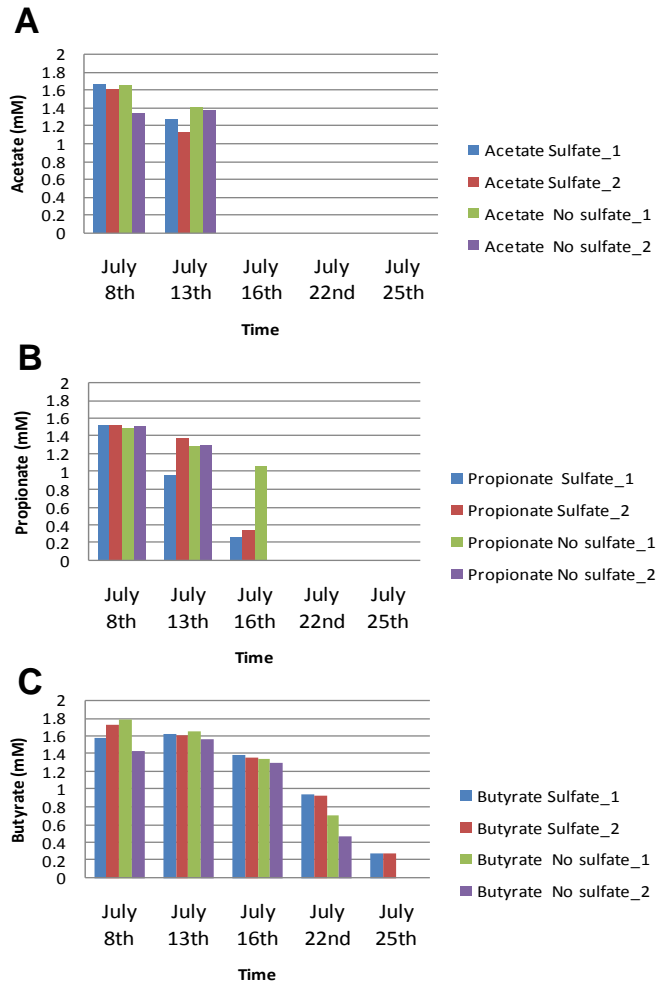
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Figure 1. Experimental flow chart



Activities and community structure of methanogenic archaea were explored by using multiple different approaches, including microcosm and molecular analyses.

Figure 2. Organic acid uptake in whale-fall sediment microcosms

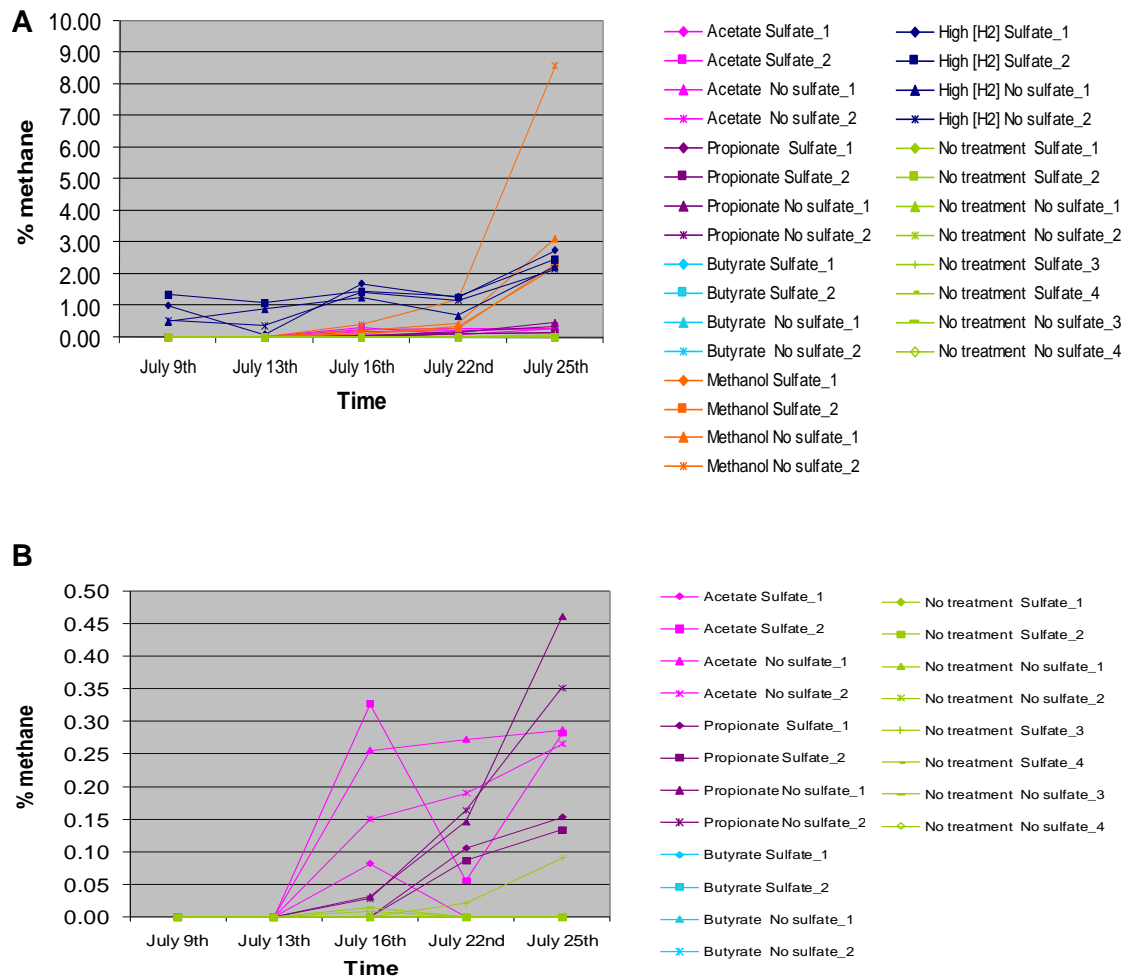


A. Acetate uptake in microcosms 1 (Acetate Sulfate_1), 2 (Acetate Sulfate_2), 3 (Acetate No sulfate_1), 4 (Acetate No sulfate_1) were measured by using HPLC.

B. Propionate uptake in microcosms 5 (Propionate Sulfate_1), 6 (Propionate Sulfate_2), 7 (Propionate No sulfate_1), 8 (Propionate No sulfate_1) were measured by using HPLC.

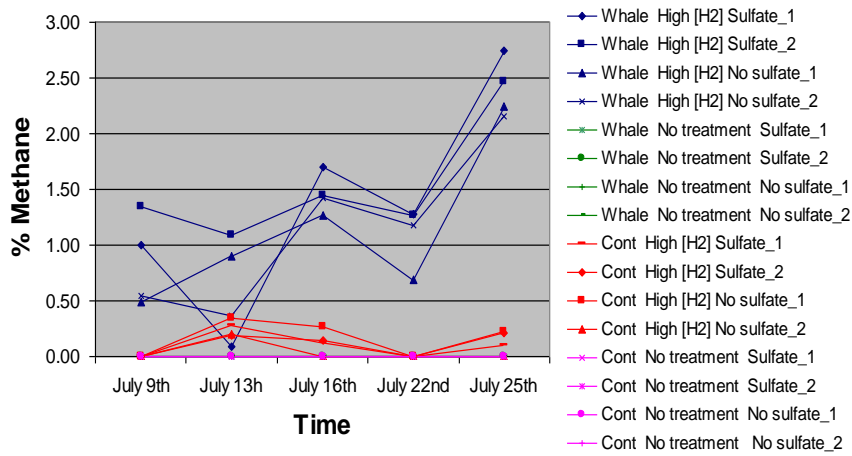
C. Butyrate uptake in microcosms 9 (Butyrate Sulfate_1), 10 (Butyrate Sulfate_2), 11 (Butyrate No sulfate_1), 12 (Butyrate No sulfate_1) were measured by using HPLC.

Figure 3. Methane production in whale-fall sediment microcosms treated with different substrates.



A. Comparing methane production in all whale-fall sediment microcosms. **B.** Comparing methane production in whale-fall sediment microcosms amended with organic acids (these data are also included to figure 3A).

Figure 4. Methane production in whale-fall and control sediment microcosms treated with high [H₂].



The increase in the methane production in response to the treatment with hydrogen gas was only observed in the whale-fall sediment.

Figure 5. Phylogenetic analysis of archaeal 16S rDNA diversity in the whale-fall sediment sample used for this microcosm study.

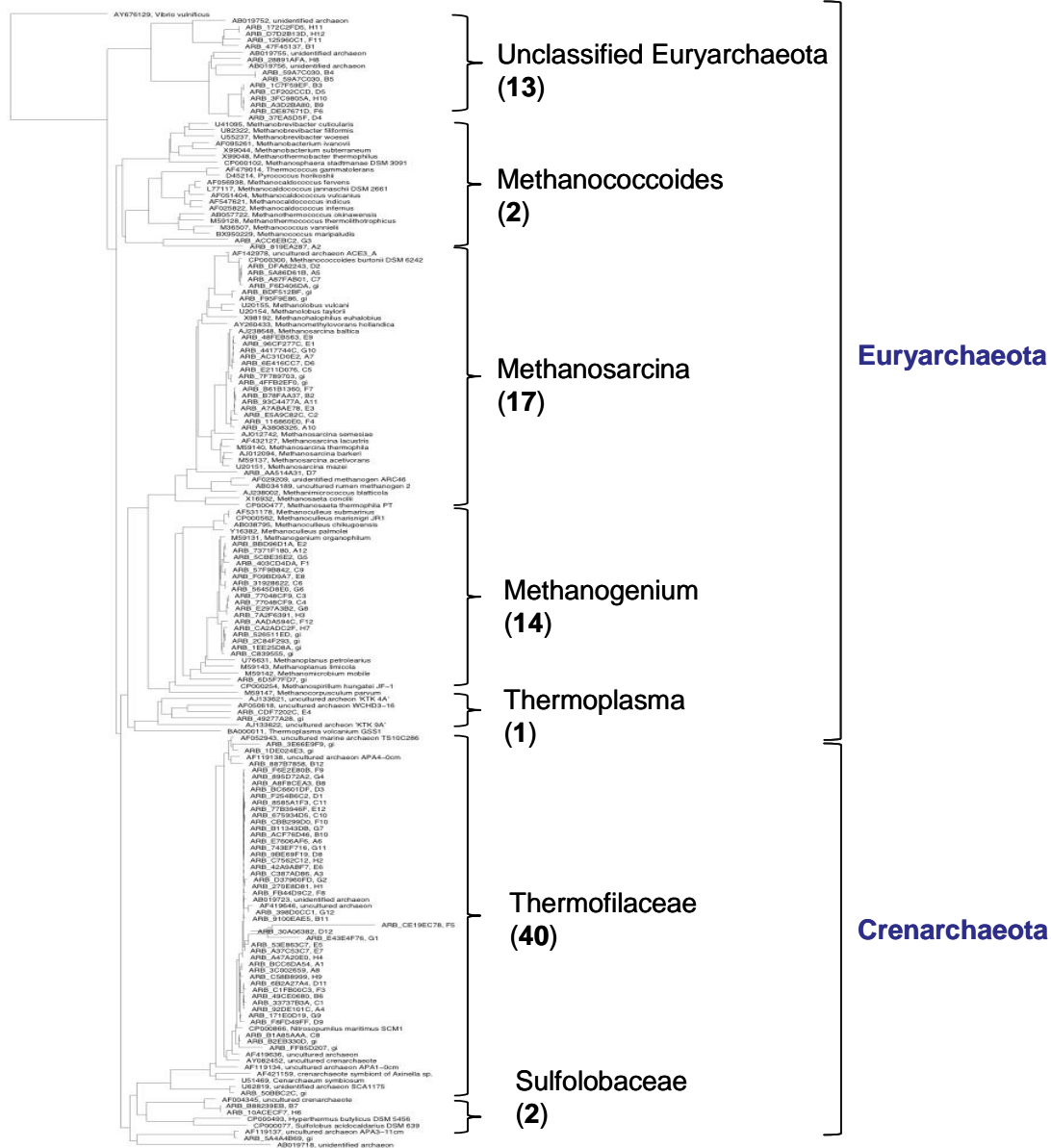
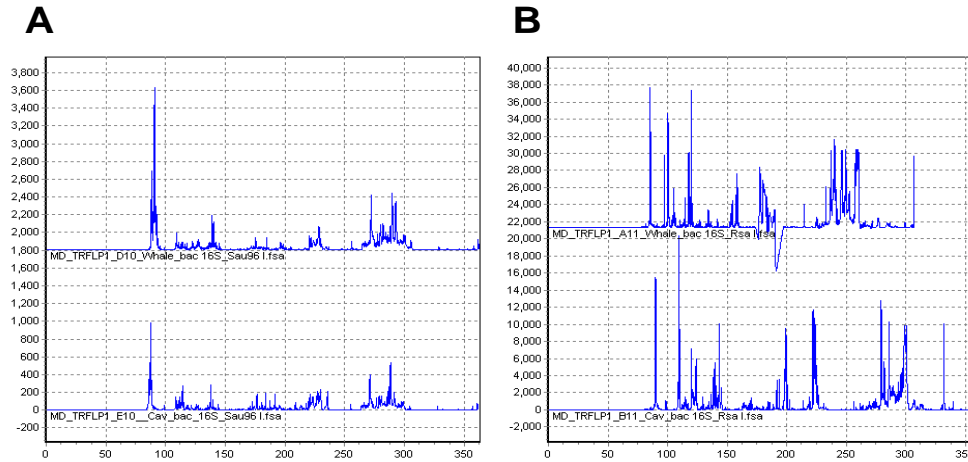
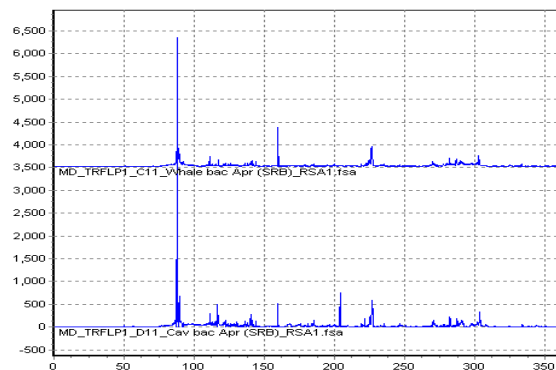


Figure 6. T-RFLP analysis of bacterial 16S rDNA diversity profile in whale-fall and control sediment samples before treatments.



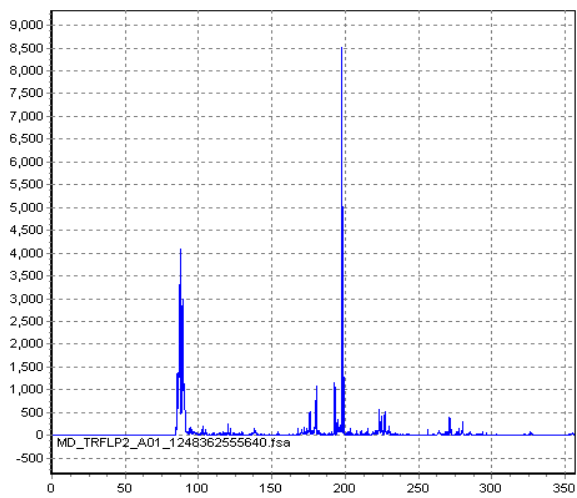
A community finger printing analysis using T-RFLP suggested that the community composition of bacteria in the whale-fall and control sediments are different each other. (A) 16S rDNA PCR product digested with Sau96 I. (B) 16S rDNA PCR product digested with Rsa I.

Figure 7. T-RFLP analysis of APS reductase gene diversity profile in whale-fall and control sediment samples before treatments.



T-RFLP analyses of *AprA* diversity profiles in the whale-fall and control sediments showed that sulfate reducing bacteria that are present in both sediments can be similar each other

Figure 8. T-RFLP analysis of MCR gene sequence diversity profile in whale-fall and control sediment samples before treatments.



T-RFLP analyses of *McrA* diversity profile was only performed for the whale-fall sediment as amplification of the gene has failed for the control sediment.

Table 1. Treatments for microcosms.

	Sediment sample	Treatment 1	Treatment 2
1	Whale-fall	Acetate	Sulfate
2	Whale-fall	Acetate	Sulfate
3	Whale-fall	Acetate	None
4	Whale-fall	Acetate	None
5	Whale-fall	Propionate	Sulfate
6	Whale-fall	Propionate	Sulfate
7	Whale-fall	Propionate	None
8	Whale-fall	Propionate	None
9	Whale-fall	Butyrate	Sulfate
10	Whale-fall	Butyrate	Sulfate
11	Whale-fall	Butyrate	None
12	Whale-fall	Butyrate	None
13	Whale-fall	Methanol	Sulfate
14	Whale-fall	Methanol	Sulfate
15	Whale-fall	Methanol	None
16	Whale-fall	Methanol	None
17	Whale-fall	High [H ₂]	Sulfate
18	Whale-fall	High [H ₂]	Sulfate
19	Whale-fall	High [H ₂]	None
20	Whale-fall	High [H ₂]	None
21	Whale-fall	None	Sulfate
22	Whale-fall	None	Sulfate
23	Whale-fall	None	None
24	Whale-fall	None	None
25	Whale-fall	None	Sulfate
26	Whale-fall	None	Sulfate
27	Whale-fall	None	None
28	Whale-fall	None	None
29	Control	High [H ₂]	Sulfate
30	Control	High [H ₂]	Sulfate
31	Control	High [H ₂]	None
32	Control	High [H ₂]	None
33	Control	None	Sulfate
34	Control	None	Sulfate
35	Control	None	None
36	Control	None	None

Microcosms with whale-fall sediment slurry were treated with different combinations of substrate that can potentially promote methanogenesis in the microcosms. Control sediment was only treated with high concentration of hydrogen with or without sulfate.