Methanogen Oxygen Stress:
An Investigation of the Superoxide Dismutase Gene

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Abstract:

Methanogenesis is a major metabolic pathway long associated with strictly anaerobic conditions predominantly due in part to the high sensitivity of obligate methanogenic archaea to oxygen toxicity. However, there is increasing evidence that methanogenic archaea can survive prolonged periods of oxygen stress in both pure culture and environmental conditions. The molecular defense mechanisms against oxygen stress in strict anaerobic microbes is unclear but there is mounting genomic evidence that complex antioxidative defense enzymes associated with aerobes may play a major role. The effect of oxygen stress on methanogenesis production, the presence/absence of the SOD gene, and Archaea community structure was investigated using a soil slurry incubation experiment with inoculum from two Trunk River soil types. The growth of archeal communities was approximated by measuring methane production rates from incubation serum vials. DNA was extracted for SOD analysis and TOPO cloning at regular intervals before and after oxygen stress to determine any subsequent shift in the archeal microbial population or SOD gene expression. It was found that methane production was reduced significantly after oxygen stress but returned to near control levels when reduced conditions were reestablished. Lastly, SOD was detected in meso-enrichment cultures, as well as, the positive methanogenic control and is likely present in populations that survived oxygen stress but due to the primers utilized in this study can't be explicitly linked to methanogens.

Introduction

Methanogenesis is a major metabolic pathway associated with strictly anaerobic conditions predominantly due to 1) the high sensitivity of obligate methanogenic archaea to oxygen toxicity and the 2) the low energy yield of this pathway. Methyl coenzyme M reductase (MCR), the last enzyme in the methanogenesis pathway is one of many methanogenic proteins to undergo rapid degradation in the presence of oxygen (Nagle and Wolfe 1983). Oxygen exposure for this specific enzyme results in the permanent inactivation of the reduced nickel (Ni (I))-tetrapyrrole activation center (Cedervall et a. 2010). The loss of MCR function completely inhibits the growth of methanogenic communities and halts methane production. In addition, the principle electron acceptor of methanogenesis, carbon dioxide, generates less energy per mol compared to all other commonly used electron acceptors (Hedin et al. 1998, Canfield et al. 2004). Archeal methanogens are therefore energetically outcompeted by almost all other microbial functional groups limiting their distribution to extremely reduced environments where more favorable electron acceptors such as oxygen, nitrate, or sulfate are unavailable?? (Conrad 1996).

Methanogens are viewed as organisms that can't tolerate oxygenic conditions due to the loss of metabolic capabilities and an inability to compete with aerobic organisms that utilize more energetically favorable pathways. However, there is increasing evidence that methanogenic archaea can survive prolonged periods of oxygen stress in both pure culture and environmental conditions (see Table 1). This survival occurs despite the rapid degradation of methanogenic proteins, like MCR. Upon the re-establishment of anoxic conditions, methanogens that have undergone oxygen stress can also rapidly grow and produce methane (Brioukhanov et a. 2006). This resilience was first observed in managed rice paddy soils which experience regular patterns of flooding and drying (Peters and Conrad 1990). Yuan et al. 2011 demonstrated via qPCR that the abundance of methanogens was reduced one log after twenty four hours of oxygen stress but that the community persisted after seventy two hours of oxygen stress. Low-level methane production has also been demonstrated in desert soils under oxic conditions (Angel et al. 2011).

The molecular defense mechanisms against oxygen stress in strict anaerobic microbes is unclear but there is mounting genomic evidence that complex antioxidative defense enzymes associated with aerobes may play a major role (Brioukhanov and Netrusov 2007). These enzymes bind to super oxide, hydrogen peroxide, and hydroxyl radicals which are products of auto-oxidation of reduced iron-sulfur proteins, flavoproteins, and quinones in response to oxygen. Other lines of defense such as cellular community floccing and soil associations may also be prevalent but will not be examined in this investigation. Superoxide dismutase (SOD) is the most prevalent antioxidant enzyme found in anaerobic organisms targeting both superoxide and hydrogen peroxide (Tholen et al. 2007). The activity of SOD measured by colorimetric assay has been associated with methanogens such as *Methanobrevibacter arboriphilus, Methanobrevibacter cuticularis,* and *Methanosarcina barkeri* that can temporarily tolerate atmospheric levels of oxygen (Tholen et al. 2007). Brioukhanov et al. 2006 demonstrated increased production of SOD mRNA in *Methanosarcina barkeri* after two hours of exposure to both hydrogen peroxide and paraquat with increased expression in response to greater concentrations of either oxidative agent. Further, the expression of SOD decreased after hydrogen peroxide and paraquat concentrations decreased.

The objective of this investigation is to determine if methanogens from Trunk River, MA are capable of surviving extended periods of oxygen stress. This will differ from previous

studies in that environmental samples with a large consortium of methanogens will be examined as opposed to single species isolation experiments. The results will give some insight as to whether oxygen defense mechanisms, namely superoxide dismutase expression, are relevant in an ecological system, albeit in controlled mesocosm experiments. To examine potential oxygen stress in methanogenic communities, two soil types: muddy and sandy, will be collected from Trunk River. The sandy soil is exposed to greater oxygen concentrations compared to the muddy soil due to its larger pore size, as well as its closer proximity to ocean's oxygenic flushing forces. I hypothesize that:

1) **Communities from both the muddy and sandy soil will be resilient to oxygen stress observed through continued methane production. Each soil type supports large natural methanogenic communities and there is a high likelihood that a subset of organisms in the environment will contain oxidative stress defenses.**
2) **The sandy soil will have greater presence of SOD as communities in this soil type are regularly exposed to higher levels of oxygen than muddy soil.**

Both before and after oxygen stress DNA will be extracted from each soil type to examine SOD presence, community analysis via TOPO cloning, and methanogenic abundance via MCR qPCR.

Approach

The effect of oxygen stress on methanogenesis production, the presence/absence of the SOD gene, and Archaea community structure was investigated using a soil slurry incubation experiment with inoculum from two Trunk River soil types. The growth of archeal communities was approximated by measuring methane production rates from incubation serum vials. When growth reached an apparent exponential phase, a twenty four oxygen stress was started followed by re-reduction of the slurry . Slurry samples were removed and DNA was extracted for SOD analysis, mcrA qPCR, and TOPO cloning at regular intervals before and after oxygen stress to determine any subsequent shift in the archeal microbial population or SOD gene expression.

Research Site and Sample Collection

The research site is located at the mouth of Trunk River in close approximately to the Shining Sea Bikeway that connects Woods Hole to Falmouth, MA. 100 meters upstream from the mouth of Trunk River three environmental samples were taken from both muddy and sandy sediment (see Fig. 1). Samples were collected by digging a small hole approximately 0.5 meters in depth and collecting sediment in a 500mL mason jar. The jars were closed underneath the water level to keep collected samples anaerobic. A small subset of both the muddy and sandy soil types was immediately extracted for DNA with MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA) for 454 analysis.

Incubation Setup

Media was prepared to approximate the salinity of the brackish water (salinity 4-8 ppt) found at Trunk River. The specific recipe is:

1. Add 0.1mL of 800mL of Freshwater Base (FWB) and 200mL of Saltwater Base (SWB) Media. Add:
   - 20 mL of 1M MOPS (pH 7.2)
   - 1.0 mL 0.1M potassium phosphate (pH 6.8)
   - 1 mL 1000X trace elements solution

- 1 mL 1000X vitamin mix
- 0.7 g Sodium Acetate TriHydrate

2. Bubble with nitrogen gas for one hour
3. Bring into anaerobic chamber. Add:
   - 70 mL 1M $NaHCO_3$
   - 10 mL 0.5M NH_4Cl
   - 2.0 mL 0.2M cysteine-HCL
   - 2.0 mL 0.2M Na_2S
   - 2.5 mL methanol
4. Add 35mL of media to fourteen 160mL serum vials, cap with blue septum, crimp
5. Remove from chamber, autoclave
6. Add 86uL of rifampicin

Ten grams of muddy soil sample #1, #2, and #3 and ten grams of sandy soil sample #1, #2, and #3 were added to two vials each under a stream of nitrogen gas. Two additional sample vials had no sample added and served as negative controls. Each sample was then flushed with 80% N_2/20% H_2 gas and then over pressurized for 8 seconds. All three common methanogenesis electron donors were added to each vial in combination with rifampicin to strongly stimulate methanogenic communities to foster enough growth in the short timeframe of the investigation. In total there were fourteen vials. All vials were incubated in the dark at 30°C.

Methane Measurements and oxygen stress

The concentration of methane gas from the headspace of each incubation vial (n=14) was measured every 24 hours for ten days with a flame ionization detector (FID) of a Shimadzu GC 2014 (Shimazdu Scientific Instruments, Columbia, MD). Concentration was determined by

Area Count*(1 atm/770000000)*115mL$^{\#}$*(1/24mL$^{\#\#}$)*(1/0.045mL$^{\#\#\#}$)

$^{\#}$115mL = Headspace
$^{\#\#}$ (1/24mL) = mmol methane occupies at 25°C
$^{\#\#\#}$45mL = media + inoculum (or amount of culture)

When each sample type generated methane production rates that resembled exponential growth (at 67 hours for muddy samples and at 117 hours for sandy soil type) one replicate from each environmental sample designated for oxygen stress was:

- Flushed with house air for 12 minutes
- Over pressurized with house air for 8 seconds
- Incubated at 30°C for twenty four hours
- Flushed with N_2 gas for 12 minutes
- Over pressurized with 80% N_2/20% H_2 gas for 8 seconds
- Anaerobically add 2.0mL of 2M Na_2S (to re-reduce the solution)

One replicate from each environmental sample designated as a control underwent the following protocol (at 91 hours for muddy samples and 141 hours of sandy soil type) and to mimic the previous flushing protocol performed on the oxygen stress treatment.

- Flushed with N_2 gas for 12 minutes
- Over pressurized with 80% N_2/20% H_2 gas for 8 seconds

- Anaerobically add 2.0mL of 2M Na_2S (to re-reduce the solution)

DNA Extraction

Every twenty four hours a 1mL aliquot of soil slurry was removed from each vial. Samples were spun for 1 min at 10,000 p.m. and the supernatant was removed. DNA was extracted from the remaining soil/cell pellet using the Power Soil DNA Extraction Kit (Carlsbad, CA).

DNA Analysis #1 - 454

The extracted DNA from each sample type was amplified with the standard 454 PCR protocol for community analysis. Approximately 43.5 ng and 28 ng of DNA were amplified from the muddy sample #2 and sandy sample #2 respectively. The PCR master mix formula is given below:

Ingredient	per sample (uL)
Phusion 2X HF MasterMix	15
DMSO, 100%	2.4
Reverse Primer, 25uM	0.6
Forward Primer, 6.25 uM	2.4
Template DNA 40-100 ng	8
Water	1.6

When the sequence reads were received the data was clustered, assigned taxonomy, and assigned otus utilizing Qiime. Subsequent data was used to make representative pie charts of the community.

DNA Analysis #2 – TOPO CLONING

The extracted DNA from muddy sample #2 designated for oxygen stress treatment at 50 hours (before oxygen stress) and 98 hours (after oxygen stress) were used for TOPO cloning. A before and after oxygen stress treatment of the sandy soil was not done due to the time it took these samples to reach their exponential growth phase and the short timeframe of the project. Extracted DNA was amplified with 16s archeal primer with the PCR master mix formula and protocol given below:

Ingredient	per sample (uL)
2X Master Mix	12.5
Forward Primer, 10uM	1
Reverse Primer, 10uM	1
Template DNA ~10ng	2
Water	13.5

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	5 min	1X
Denaturation	95	30 sec	30X
Annealing	46	30 sec	
Extention	72	90 sec	
Final Extension	72	5 mins	1x
Hold	4	-	

The amplified product (n=2) was ligated and transformed according to the protocol below:

1. Add 1uL of pCR4-TOPO vector, 1uL of diluted slate and 3uL of PCR product to eppendorf tube
2. Incubate 2 hours at room temperature
3. Add each reaction to one tube of thawed (on ice) component cells
4. Stir gently with pipette tip
5. Transfer entire mixture to electroporation cuvette
6. Electroporate cells with following conditions (voltage = 2250 V, resistance – 200 Ohm, capacitance = 25 uF)
7. Add 250uL of room temperature SOC to each cuvette
8. Pipette entire cell mixture back into component tube
9. Incubation at 37°C for 1 hour with shaker (~200 rpm)
10. Transfer 60uL of cell mixture to 60uL of SOC.
11. Pipette 100uL onto one plate (amp+) and 10uL onto another (amp+)
12. Mix with beads, place at 37°C for 16 hours
13. Pick colonies and place into 96 well plate with amp+ media, grow overnight in shaker (250 rpm)

The grown cells were submitted for Sanger sequencing and the subsequent sequences were again analyzed with Qiime to build representative pie charts at the genus level.

DNA Analysis #3 – qPCR

The extracted DNA from muddy sample #2 [control days 2-7 and oxygen stress days 2-7], sandy sample #2 [control days 2-8 and oxygen stress days 2-8], and a positive control from *Methanospirillium* pure culture were amplified for e mcrA gene using the following master mix and PCR protocols following the protocol from Steinberg and Regan 2009.

Total n = 27

Ingredient	per sample (uL)
2X Master Mix	25
Forward Primer* (10uM)	2.5
Reverse Primer** (10umM)	2.5
Template DNA (2.5 ng/uL)	1
Water	19

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	3 min	1X
Denaturation	95	30 sec	30X
Annealing***	48	45 secs	
Extention	72	30 secs	
Denaturation	95	30 secs	30X
Annealing	55	45 secs	
Extention	72	30 secs	
Final Extension	72	15 mins	1x
Hold	4	-	

*mlas forward primer 5' – GGTGTGTMGGDTTCACMCARTA – 3'
**mcrA reverse primer 5' – GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC – 3'
*** ramp of 0.1°C per second

The *Methanospirillum* control ng/ul was calculated with the following formula.

Copy #/ng = (6.022*1023 cells/mol) * (1/497bp) * (1/650 g/mol) * (1/1e9 ng/g)
Copy#/ng = 1.8641*10^9
Copy/uL = 1.8641 *10^9 * 55.4 ng/uL = 1.03272 *10^11 copy/uL

It was then serially diluted to 1*10^5, 1*10^4, 1*10^3, 1*10^2, 1*10^1, with 2uL/ng irradiated sheared salmon sperm. The qPCR master mix is given below.

Ingredient	per sample (uL)
2X QauntiFast SYBR Green	12.5
Forward Primer* (10uM)	1.25
Reverse Primer** (10umM)	1.25
Template DNA (undiluted)	1
Water	9

PCR reaction was amplified with StepOne Plus Real Time PCR System (Applied Biosystem) with the Real-time two-step fast reaction given below:

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	5	1X
Denaturation	95	10 secs	35
Annealing	60	30 secs	

DNA Analysis #4 – SOD gene amplification

The objective for the design of the SOD primer (Fe-C) was to create degenerative primers that could both 1) capture the entire methanogenic community and 2) sustain robust

amplification because thorough PCR optimization for this project was not feasible. The following degenerative primers were created with the following process:

Primers
>superoxide dismutase (Fe-C) forward_1(A1) 50ng
GAAGAACAATTAAGAATACATCATVMNAARCAYCA
>superoxide dismutase (Fe-C) modforward_1(A1) 50ng
GAAGAACAATTAAGAATACATCATVMNAARBRYCA
>superoxide dismutase (Fe-C) forward_2(A2) 50ng
TCTGAAGAACAATTAAGAATACATCAYVMNAARCA
>superoxide dismutase (Fe-C) modforward_2(A2) 50ng
TCTGAAGAACAATTAAGAATACATCAYVMNMRRCA
>superoxide dismutase (Fe-C) forward_3(A3) 50ng
TATATATCTGAAGAACAATTAAGAATACAYCAYVMNAA
>superoxide dismutase (Fe-C) modforward_3 (A3) 50ng
TATATATCTGAAGAACAATTAAGAATACAYCAHVVNMA
>superoxide dismutase (Fe-C) reverse_1 (C15) 50ng
TCAGTCATCTTACAATAAGTTAAARCNSCCCANCC
>superoxide dismutase (Fe-C) modreverse_1 (C15) 50ng
TCAGTCATCTTACAATAAGTTAAARCNSCMCMNMC
>superoxide dismutase (Fe-C) reverse_2 (C20) 50 ng
TCTAAAATCTGGATATACATTAACATTRTGYTTYTC
>superoxide dismutase (Fe-C) modreverse_2 (C20) 50 ng
TCTAAAATCTGGATATACATTAACATTRBSNYTHTC
>superoxide dismutase (Fe-C) reverse_3 (C22) 50 ng
GCTCTATCATTCTTATAATCTATATAATAAGCRTGYTCCCA
>superoxide dismutase (Fe-C) modreverse_3 (C22) 50 ng
GCTCTATCATTCTTATAATCTATATAATAARCRTGYTYNCA
Protocol:

1. Download amino acid sequences of SOD (Fe-C) from 17 listed methanogens (www.uniprot.org)
2. Align and translate sequences and create primers with “core” and “clamp” regions using www.pprospector.net
3. Introduce further degeneracy by introducing degenerative bases near the 3’ end to facilitate amplification
4. Run multiple annealing temperature gradient (96 rxn) PCRs to evaluate primers pairs to select the best one

The primer pair F1/R3mod produced the strongest bands at the highest annealing temperature (52°C). From this data (see Fig. 2) the following PCR master mix and protocol were optimized.

Ingredient	per sample (uL)
2X Master Mix	25
Forward Primer* (10uM)	5
Reverse Primer** (10umM)	5
Template DNA (0.5 ng/uL)	2.5
Water	12.5

Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	5 min	1X
Denaturation	95	30 sec	5X
Annealing***	42	60 secs	
Extention	72	60 secs	
Denaturation	95	30 secs	25X
Annealing	46	60 secs	
Extention	72	60 secs	
Final Extension	72	15 mins	1x
Hold	4	-	

*** ramp of 0.1°C per second

Product was visualized on 1% Agar Gel using standard loading dye (6X) and New England BioLabs 50 bp ladder.

Results and Discussion

Methane production in the sandy and muddy soil had significantly different growth rates. Exponential growth (see Fig 4.) was reached in the sandy soil after five days (measured at 113 hours). After oxygen stress average methane production in sandy soil replicates was reduced 4.5X compared to the control replicates. Upon re-reduction, average methane production was equivalent to the average methane production in the control samples within 48 hours. Exponential growth (see Fig 5.) was reached in the muddy soil after two days. After oxygen stress average methane production in the oxygen stress replicates was reduced 5X compared to the control replicates. Again, after 48 hours the average methane production of the oxygen stress treatment samples was equivalent to the methane production in the control samples. In fact, oxygen stress replicate #2 produced equivalent methane production to the control conditions within 24 hours. The wide variability observed between replicates for both soil types was most likely due to the heterogeneous nature of wetland soil types. This condition was exacerbated in the muddy soils which contained organic matter particles of varied sizes that were difficult to distribute equally amongst the replicates. The negative controls produced negligible levels of methane throughout the experiment (data not shown).

The return to normal methane production for the oxygen stress treatment in both the muddy and sandy sample types indicates that methanogenesis resumed quickly after reduced conditions were reestablished. The nature of this experiment however makes it difficult to discern whether the majority of the various methanogenic populations were able to survive and resume growth or whether a small subset of methanogens survived oxygen stress conditions and were selected for and thereby changed the structure of the community. The former scenario would result in an exponential growth curve following re-reduction while the latter scenario would result in a linear growth curve as the small selected population slowly grows. There were not enough valid time points after re-reduction to determine growth curves because the mesocosms became limited in nutrients inhibiting both growth and methane production. Further, the TOPO cloning (see Fig. 6) demonstrated a change in community structure between the before

oxygen stress population which contained a wide variety of methanogenic species to the after oxygen stress population which was dominated by *Methanosarcina* species. This data however, lacks results from the control treatment. It is possible that the methanogen enrichment itself and not the oxygen stress selected for *Methanosarcina* populations limiting the scope of the TOPO cloning data. Despite the lack of resolution to determine the type of growth that occurred following oxygen stress the data indicates that methane production quickly returned to control levels supporting my 1st hypothesis.

The qPCR (see Fig. 7) demonstrated lower abundance of the mcrA gene in the oxygen stress replicates for both soil types compared to the control treatments. Oxygen stress resulted in a two log decrease in copy number of the mcrA gene in the muddy soil type. In the sandy soil oxygen stress resulted in a log decrease but was not correlated to when oxygen stress was introduced. This data corroborates that oxygen stress reduces methanogenesis but does not indicate that methanogenesis (or at least copy #) increases when reduced conditions are reestablished. Due to time constraints of this course I had only one opportunity to run qPCR. One environmental replicate (compared to three replicates tested for methane concentration) for each soil type and treatment condition was amplified. Further, the majority of counts had to be extrapolated beyond the control curve ($r^2 = 0.994$) due to the majority of the samples having greater copy #s of mcrA than anticipated. For these reasons the qPCR was only used to corroborate data produced during methane analysis and nothing further.

Lastly, the correct size (~496 bp) SOD fragment was detected in a majority of samples for both soil types. In the sandy soil (see Fig. 7) significantly brighter bands were observed for the oxygen stress treatment (days 2-8) compared to the control treatment (days 2-8). By far the brightest band occurred on the day after oxygen stress was induced with all subsequent days after reduced conditions were reestablished having less bright bands. The positive control, the DNA extracted from pure culture *Methanospirillium,* also had a positive band. These results indicate that SOD is present and detected in methanogens (positive control) and the concentration of SOD copy number in the environment was greater after oxygen stress. The results from the muddy soil (see Fig. 8) were not as clear. Again, SOD bands were detected in the majority of samples but had no correlation to oxygen stress. The oxygen stress treatment (days 2-7) didn't have more or brighter bands compared to the control treatment (days 2-7) nor did the DNA extraction from the day of oxygen stress (day 4) have a detectable band of SOD. Overall, this data appears to support hypothesis two in that the sandy soil type had greater concentration of the SOD gene detected post oxygen stress compared to the muddy soil type . It should be noted that the other data, methane production and qPCR of the mcrA gene, didn't support this hypothesis. In both of these data sets the muddy and sandy soil type had equivalent responses to oxygen stress. With this in mind the sequences of the primer pair (F1/R3mod) used to amplify the SOD gene were compared to the microbial database of the SOD gene from img.jgi.gov. It was found that not only does this primer pair bind stringently to the SOD gene found in methanogens but also several hundred bacterial species predominantly in the bacillus family (see Fig. 9). It is possible that the increased concentration of the SOD gene observed after oxygen stress in the sandy soil is not from methanogenic populations but from dormant or less active bacterial populations that can more readily grow in oxic conditions. Overall, the data from this experiment does not support the 2nd hypothesis. The next logical step would be to sequence the SOD PCR product to determine what organism types contain this gene. This information may help link oxygen stress and SOD oxidative defense mechanism in methanogens together.

Conclusion

Methane production was reduced significantly after oxygen stress but returned to near control levels when reduced conditions were reestablished. Oxygen stress also potentially reduced the abundance of mcrA copy number and altered community composition in favor of *Methanosarcina* species. Lastly, SOD was detected in meso-enrichment cultures, as well as, the positive methanogenic control and is likely present in populations that survived oxygen stress. However, the primers utilized in this investigation can't resolve whether the SOD detected originated from methanogenic, general archeal, or bacterial populations.

Acknowledgements

I would like to thank the course directors, Dan Buckley and Steve Zinder, as well as, all of the TAs and students for providing support and advice throughout this mini-project. I would like to especially thank Suzanna Brauer for providing the inspiration for exploring the SOD gene in methanogens and the many helpful hints she had throughout the experiment. Chuck Pep-Ranney help was also instrumental as he assisted with primer design, genomic analysis, and the fantastic figure 9.

Appendix I: Figures

Table 1: Methanogenic tolerance of aerobiosis conditions

Microorganism	Conditions of Aerobiosis	Survival	Comments
Methanobrev-bacter	6-10%	Several Hours	Leadbetter et al. 1996, Tholen et al. 2006
Methanococcus voltae	20%	Up to 10 hours	Brioukhanov et al. 2007
Methanosarcina barkeri	20%	At least 24 hours	Brioukhanov et al. 2006

Table 1: Modified from Brioukhanov et al. 2006 and Brioukhanov et al. 2007. The table lists methanogenic genus that have been observed to survival oxygen stress (predominantly in pure culture experiments).

Figure 1: Image of Sampling Sites

Fig. 1: Diagram illustrates location of sandy soil (SS) sites and muddy soil (MS) sites. Each site was approximately 10 meters from its closest neighbor. If you wish to sample for this site be wary of the thigh deep muddy sediment.

Figure 2: Example of SOD PCR optimization

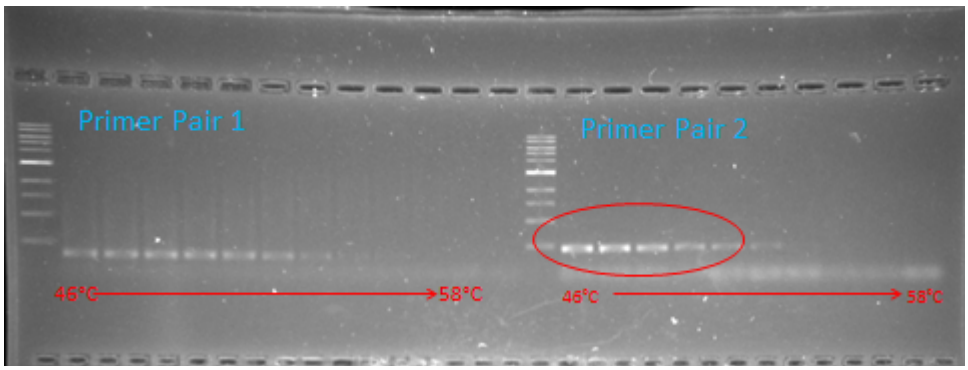

Fig. 2: Image illustrates 12 PCR reactions from two different primer pairs across annealing temperature gradient of 46°C to 58°C. Primer Pair 2 (F1/R3mod) produced the strongest bands and was used for all amplification reactions for SOD.

Figure 3: Methane Production Rate in Sandy Soil

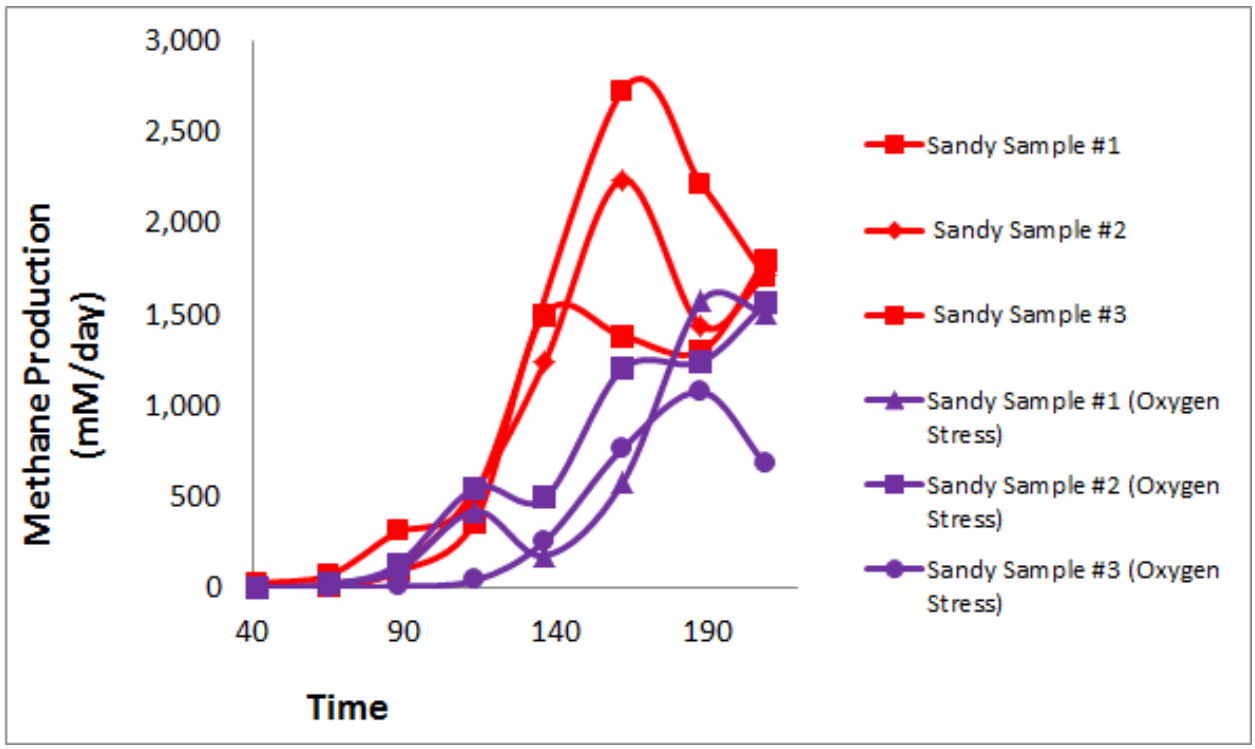

Fig. 3: Exponential growth was reached in the sandy soil after five days (measured at 113 hours). After oxygen stress average methane production in sandy soil replicates was reduced 4.5X compared to the control replicates. Upon re-reduction average methane production was equivalent to the average methane production in the control samples within forty eight hours.

Figure 4: Methane Production Rate in Muddy Soil

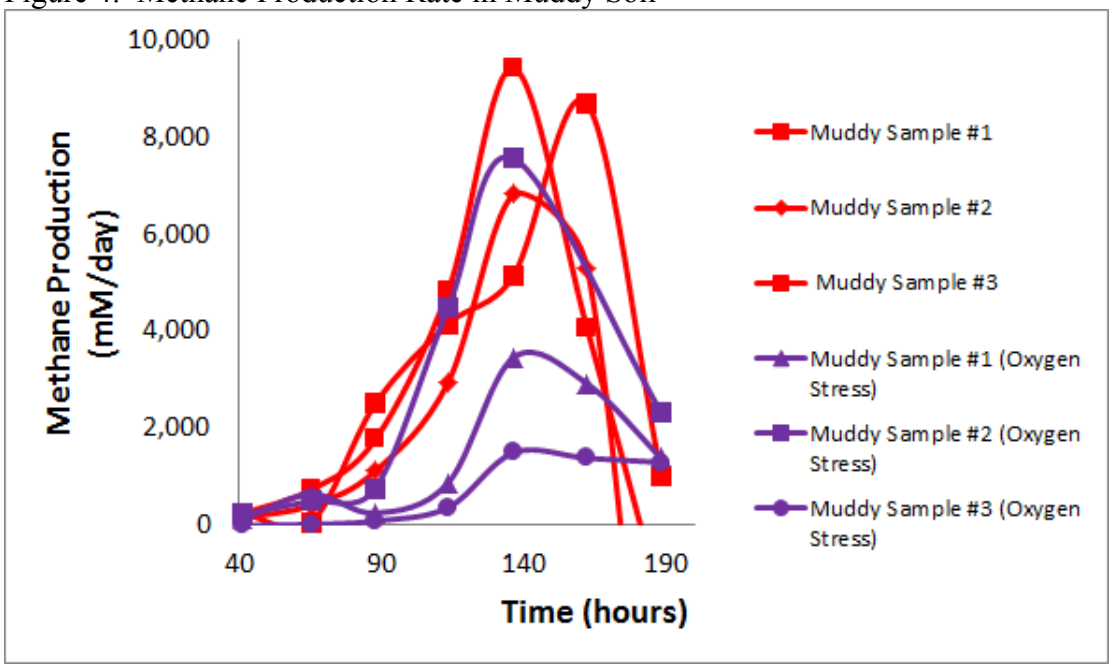

Fig 4. Exponential growth was reached after two days. After oxygen stress average methane production in the muddy soil replicates was reduced 5X compared to the control replicates. Forty eight hours after re-reduction the average methane production of the oxygen stress samples was equivalent to the methane production in the control samples.

Figure 5: Methane Production Rate in Muddy Soil

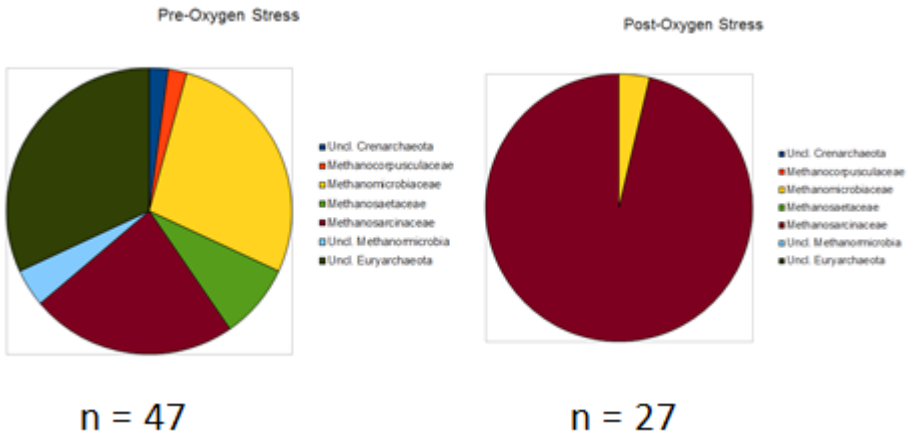

Fig 5. Pie charts demonstrated a change in community structure between the before oxygen stress population which contained a wide variety of methanogenic species to the after oxygen stress population which was dominated by *Methanosarcina* species. This data however, lacks results from the control condition. It is possible that the methanogen enrichment itself and not the oxygen stress selected for *Methanosarcina* populations limiting the scope of the TOPO cloning data.

Figure 6: qPCR of mcrA gene in both sandy and muddy soil

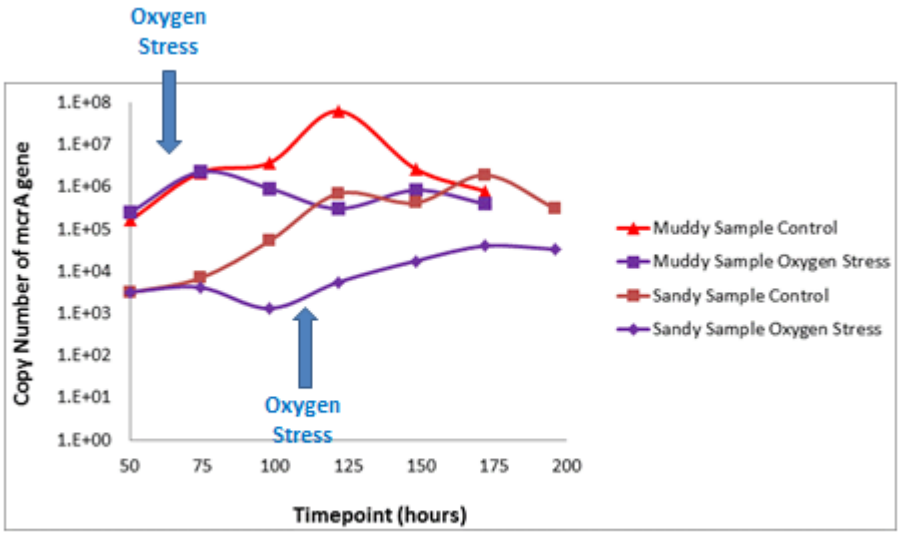

Figure 6: The qPCR graph demonstrates lower abundance of the mcrA gene in the oxygen stress replicates for both soil types compared to the control conditions. Oxygen stress resulted in a correlated two log decrease in copy number in the muddy soil. In the sandy soil oxygen stress resulted in a log decrease but was not correlated to when oxygen stress was introduced.

Figure 7: Gel Image of SOD amplification of sandy soil

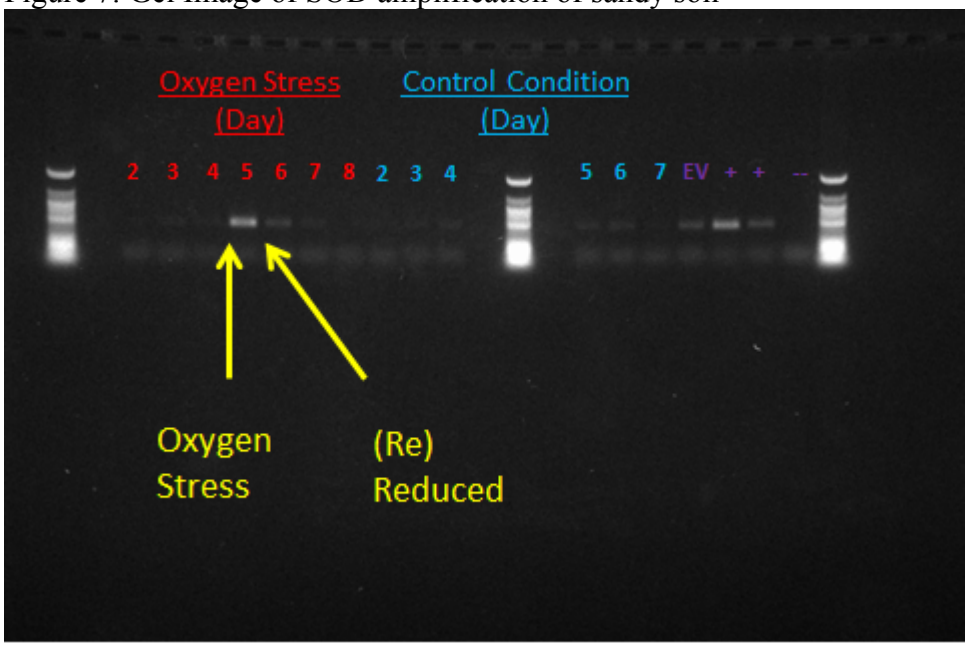

Figure 7: Significantly brighter bands were observed for the oxygen stress treatment (days 2-8) compared to the control treatment (days 2-7). By far the brightest band occurred on the day after oxygen stress was induced with all subsequent days after reduced conditions were reestablished having less bright bands. The positive control, the DNA extracted from pure culture *Methanospirillium,* labeled "+" also had a positive band.

Figure 8: Gel Image of SOD amplification of muddy soil

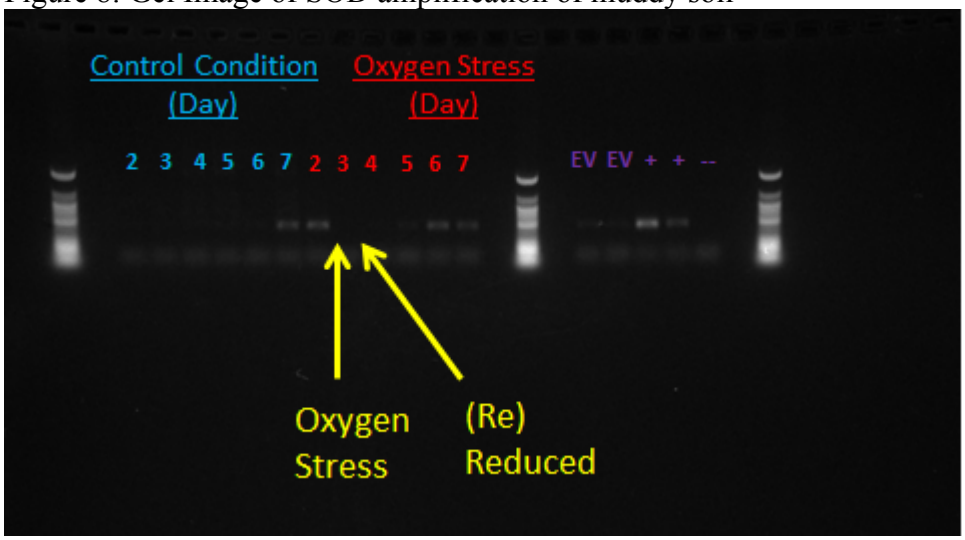

Figure 8: The results indicate no increase SOD gene concentration in samples which have experienced oxygen stress. There appears to be no correlation between SOD gene detection and treatment condition.

Figure 9: Primer compatibility tree

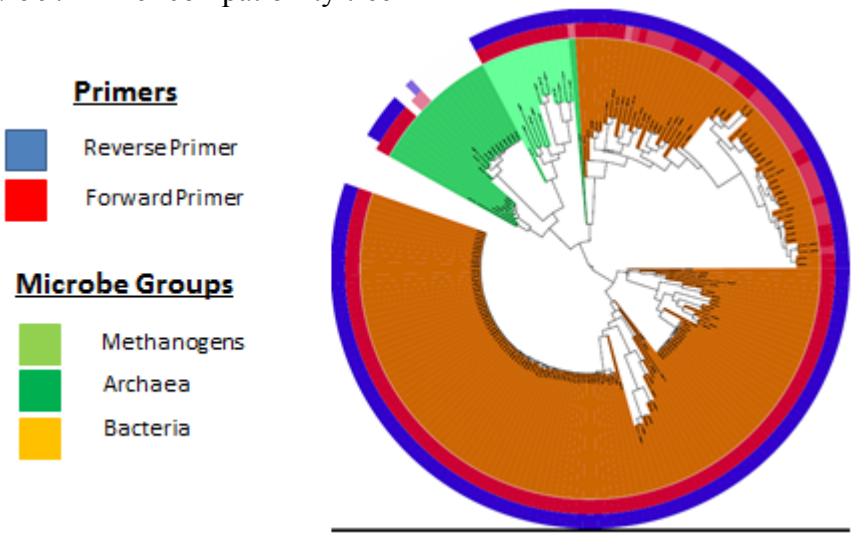

Figure 9: The sequences of the primer pair utilized bind stringently to the SOD gene found in methanogens but also several hundred bacterial species predominantly in the bacillus family

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